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Understanding the role of endothelial progenitor cells in vascular injury and repair

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Abstract

Introduction: Vascular injury is the crucial initiating event in atherosclerosis and is universal following percutaneous coronary intervention. The cellular response to this injury largely determines vessel outcome. Endothelial progenitor cells (EPCs) and their progeny, late outgrowth endothelial cells (EOCs) are thought to play an important role in this process and characterising this role would be valuable in better understanding vascular injury and repair.

Methods: The radial artery in the context of transradial cardiac catheterisation was examined as a model of vascular injury with characterisation of structural injury, longitudinal function and EPC populations. To examine the role of late outgrowth endothelial cells a method for GMP-compliant cell culture and labelling with F¹⁸Fluorodeoxyglucose was developed with a view to conducting a cell-tracking study of human administration.

Results: Radial artery function was reduced following transradial cardiac catheterisation with recovery over a period of three months. There was no correlation between recovery of arterial function and EPC populations as defined by conventional surface markers. A research grade protocol for EOC culture was successfully translated to a GMP-compliant process producing a viable, phenotypically homogeneous EOC product. Cells were successfully labelled with F¹⁸Fluorodeoxyglucose and whilst proliferation was reduced, acute viability and function were not compromised.

Conclusion: The radial artery in the context of transradial cardiac catheterisation is a useful model of vascular injury and repair although recovery of vascular function does not appear to be influenced by EPC populations. GMP-compliant culture and labelling of EOCs is feasible and will allow examination of the physiology of these cells *in vivo* in man.

Lay summary

Damage to the lining of blood vessels is a widespread problem and is the root cause of many diseases such as heart attacks and strokes. It is thought that there is a population of cells which circulate in the blood and repair these injured areas of blood vessels. A better understanding of the process of blood vessel injury and repair and the role that stem cells play would be valuable.

We studied the function of blood vessels in patients having coronary angiograms (which involves putting a catheter into one of the arteries in the wrist and causing mechanical damage) to document the process of blood vessel injury and repair and to examine the influence that stem cells have on this process.

We also developed a method to grow stem cells in a clean room environment which are fit for human administration and a process for labelling these cells with a radioactive label. This will allow us to give these cells to patients with injured blood vessels and to track their behaviour in order to determine whether they home to and repair areas of injury in blood vessels.

We hope that this will lead to a greater understanding of the processes of blood vessel repair and possibly to a therapeutic trial of these stem cells for human cardiovascular disease.

Declaration

This thesis is an original report of my own research which I conducted between August 2013 and August 2016 at the Centre for Cardiovascular Sciences at the University of Edinburgh and in the Cardiology department at the Royal Infirmary of Edinburgh.

I have been involved in all parts of the work with the exception of final clean room culture work which was carried out by staff of the Scottish national blood transfusion service as was the flow cytometry on the resultant cells described in chapter 6. All studies were approved by the South-east Scotland Research Ethics Committee and all research was conducted in accordance with the declaration of Helsinki. Written consent was obtained from each participant prior to entry into studies.

The work was supported by a project grant from the British Heart Foundation.

This work has not been submitted in support of another degree or qualification. Publications arising from this thesis are referenced at the beginning of the appropriate chapters.

Andrew J Mitchell

12/03/2018

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ABBREVIATIONS

| | |
|---------------------------|--|
| ANOVA | Analysis of variance |
| CPC | Cardiac progenitor cell |
| CD | Cluster of differentiation |
| CI | Confidence intervals |
| CR | Coefficient of repeatability |
| CT | Computerised tomography |
| DAPI | Diamidino-2-phenylindole |
| DNA | Deoxyribosenucleic acid |
| EBM | Endothelial basal medium |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| ECFC | Endothelial cell-forming cell |
| EC-CFU | Endothelial cell colony forming unit |
| EEL | External elastic lamina |
| EOC | Endothelial outgrowth cell |
| EPC | Endothelial progenitor cell |
| ¹⁸F-HFB | ¹⁸ F-Hexadecyl-4-fluorobenzoate |
| ¹⁸F-FDG | ¹⁸ F-Fluorodeoxyglucose |
| ¹⁸F-FLT | ¹⁸ F-Fluorothymidine |
| FMD | Flow-mediated dilatation |
| GCSF | Granulocyte colony-stimulating factor |
| GMP | Good manufacturing practices |

| | |
|--------------|-------------------------------------|
| HIV | Human immunodeficiency virus |
| IEL | Internal elastic lamina |
| IL | Interleukin |
| KDR | Kinase-domain receptor |
| LDL | Low-density lipoprotein |
| MBq | Megabecquerels |
| NMD | Nitrate-mediated dilatation |
| OCT | Optical coherence tomography |
| PCI | Percutaneous coronary intervention |
| PBS | Phosphate-buffered saline |
| PET | Positron-emission tomography |
| RNA | Ribonucleic acid |
| RPM | Revolutions per minute |
| SPECT | Single positron-emission tomography |
| VEGF | Vascular endothelial growth factor |

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Chapter 1

Introduction

1.1 INTRODUCTION

Vascular injury is the central mechanism in the initiation, progression and clinical consequences of atherosclerosis. Damage to blood vessels may arise either directly as a result of the disease process itself or following mechanical disruption induced by interventional procedures such as surgery or angioplasty.

The regenerative response of the vasculature is critical to vessel outcome following injury and determines the risk of events such as restenosis, plaque growth, thrombosis and negative remodelling. Reconstitution of the endothelial monolayer is a crucial step in vessel repair and it is likely that endothelial cells play an important modifying role in the response to vascular injury. Improving our understanding of the cellular mechanisms underlying endothelial regeneration may allow us to modify this process, develop novel therapies for atherosclerosis and optimise existing surgical and endovascular interventions.

1.2 VASCULAR INJURY AND REPAIR IN SPECIFIC CIRCUMSTANCES

1.2.1 Atherosclerosis

Vascular injury in atherosclerosis is characterised by lipid and inflammatory cell infiltration into the vessel wall (Gerrity 1981). Plaque progression and

clinical outcome are determined by a complex interplay between both systemic (such as blood pressure, serum cholesterol, smoking) (Libby 2013) and local (such as cellular and rheological) (Hahn *et al.* 2009) factors. Whilst atherosclerosis is predominantly a disease of the intima, there are also important contributions from the vascular media and adventitia.

1.2.1.1 Intima

The endothelium has many roles in the maintenance of vascular homeostasis. Alterations in endothelial function occur early in the atherosclerotic process, often preceding clinically detectable disease and have repeatedly been shown to predict burden of vascular disease (Tura *et al.* 2013; Phinikaridou *et al.* 2013; Simova *et al.* 2010).

Disruption of cell-cell connections and increased vascular leak is observed in the normal ageing process associated with arterial stiffening (Huynh 2011). This process is accelerated in the presence of vascular injury and leads to further alterations in vascular structure and function. For example, the permeability of the endothelium to circulating cells and biologically active macromolecules increases early in the pathophysiology of atherosclerosis, with cholesterol ingress playing a major role. In apolipoprotein E deficient mice, increased endothelial permeability correlates with disturbed gap junction structure and cholesterol infiltration on electron microscopy (Phinikaridou *et al.* 2013).

As with cholesterol, inflammatory cell infiltration of the vessel wall is believed to play a key role in atherosclerosis. Circulating monocytes migrate into the plaque where transformation into tissue macrophages is associated with uptake of oxidised low-density lipoprotein (LDL) particles and ultimately transformation into “foam cells”. Foam cells have a powerful chemotactic effect on vascular smooth muscle cells. Whilst subsets of monocytes have been described (Huynh 2011; Grage-Griebenow *et al.* 2001), the importance of these distinct populations in arterial disease has only recently been explored.

Monocytes can be subdivided into distinct populations described as ‘classical’ (~90%; CD14⁺⁺/CD16⁻) and non-classical (CD14_{Low}CD16⁺⁺). While the relative contributions of these two cell populations to plaque biology remains to be defined, classical monocytes are thought to promote local inflammation through phagocytosis and cytokine production. In contrast, non-classical monocytes may have an anti-inflammatory role through collagen deposition and plaque stabilisation (Jaipersad *et al.* 2014).

One major consequence of atherosclerotic change in the arterial wall is a predisposition to thrombosis. In health, the endothelium secretes various anti-thrombotic substances such as nitric oxide, prostacyclin and thrombomodulin as well as the pro-coagulant von Willebrand factor and plasminogen activator inhibitor. Imbalances in these pro- and anti-coagulant factors accompany

vascular injury and endothelial dysfunction, favouring a pro-thrombotic phenotype (Newby *et al.* 1999; Lang *et al.* 2008).

1.2.1.2 Media

Whilst structural changes in the media are not prominent in atherosclerosis, functional changes in vascular smooth muscle cells and their migration into the intima are. Early in atherosclerosis, vascular smooth muscle cells migrate into plaque and, depending upon their phenotype, exert both positive and negative effects on remodelling. Initially, vascular smooth muscle cells were thought to divide themselves between fibroproliferative cells, secreting collagen and other extracellular matrix proteins, and contractile cells which are neither proliferative nor secretory (Chistiakov *et al.* 2015). This is now believed to be over-simplistic and broad phenotypic differences within the fibroproliferative population are likely to result in both pro and anti-inflammatory influences (Kolb *et al.* 2007). The phenotypic profile of the vascular smooth muscle population is determined by autocrine, paracrine and mechanical factors, comprehensively reviewed by Berk and colleagues (Berk 2001). As the largest producer of vascular endothelial growth factor (VEGF) in the atherosclerotic plaque (Ho-Tin-Noe *et al.* 2011), vascular smooth muscle cells promote vessel ingrowth from the adventitia, providing a conduit for inflammatory cell ingress and a mechanism for plaque haemorrhage; processes central to progression of the atherosclerotic lesion.

1.2.1.3 Adventitia

The adventitia was previously thought to be an inert matrix of connective tissue supporting the vasculature with little role in the pathophysiology of atherosclerosis. However, there has been a recent change in this paradigm. In contrast to the traditional 'inside-out' model based around intimal inflammation, an 'outside-in' model of atherogenesis has been proposed with adventitial fibroblast activation and differentiation into myofibroblasts occurring as an early event (Scott *et al.* 1996). These latter cells migrate into the media and generate reactive oxygen species, which when present in excessive concentrations are associated with cellular damage. Increased adventitial fibroblast activation and peri-vascular lymphocyte infiltration occurs in both hypertension and atherosclerosis and precedes alterations in endothelial function (Haurani *et al.* 2007).

The importance of perivascular fat to arterial biology is increasingly recognised (Antonopoulos *et al.* 2016; Margaritis *et al.* 2013) with abnormalities seen on Positron-emission tomography-computed tomography (PET-CT) imaging both preceding clinically apparent atherosclerotic disease and reflecting instability and vascular inflammation in established atherosclerotic disease (Antonopoulos *et al.* 2017). Whilst it is unlikely that the adventitia and perivascular fat alone

initiate atherogenesis, they are not the inert connective tissue layer they were once thought to be.

1.2.1.4 Plaque rupture

Rupture of atherosclerotic plaques with associated thrombus formation is the main cause of myocardial infarction and sudden death in patients with coronary artery disease (Libby 2013). Thrombosis and intra-plaque haemorrhage are also important mechanisms by which lesions increase in volume and stenosis progresses (Silvestre-Roig *et al.* 2014).

Rupture events are most likely to occur in plaques with a large lipid-rich necrotic core and thin fibrous cap (Stone *et al.* 2011), usually at areas of foam cell aggregation (Richardson *et al.* 1989). Exposure of the lipid-rich, highly thrombogenic core to the vessel lumen leads to thrombosis on the surface of the plaque. Endothelial injury and denudation is the norm under the thrombus but it is unclear whether this precedes plaque rupture or is a consequence of the thrombotic event. Following plaque rupture and thrombosis the cascade of cellular events is largely similar to that seen in mechanical intervention (**Figure 1**) with platelet and neutrophil influx into the vessel wall as an early event (Farb *et al.* 1996). This is followed by a more chronic inflammatory response involving macrophage, lymphocyte and vascular smooth muscle cell infiltration (Bentzon *et al.* 2014). Organisation and incorporation of the thrombus into the

vessel wall with reconstitution of the fibrous cap and endothelial cell layer leads to plaque growth and potentially, a reduction in lumen calibre.

1.2.1.5 Defective repair mechanisms and plaque progression

Inflammation promotes plaque instability and progression but also attenuates the normal mechanisms of repair. Re-endothelialisation, crucial to vessel healing and repair is inhibited by a pro-inflammatory cytokine profile and promoted by an anti-inflammatory profile (Padfield *et al.* 2010). Early efforts to modify this inflammatory process suggested potential benefits with both glucocorticoid and anti-tumour necrosis factor alpha therapy (Silvestre-Roig *et al.* 2014) inhibiting atherosclerosis in pre-clinical models. Previously, these benefits seen in pre-clinical work have failed to translate into the clinical arena, with a lack of cardiovascular efficacy in trials using these drugs with licensed indications for rheumatological or dermatological conditions (Ryan *et al.* 2011).

This however changed with the publication of the CANTOS trial (Ridker *et al.* 2017) in which high risk patients with previous myocardial infarction were treated with Canakinumab, a monoclonal antibody targeting the pro-inflammatory cytokine IL1 β . There was a significant reduction in vascular events in treated patients. This effect was independent of cholesterol level which was unaffected by Canakinumab and reinforces the importance of inflammation as a driver of atherosclerosis.

1.2.2 Mechanical trauma

With the ever-expanding use of endovascular intervention, iatrogenic vascular injury has become an increasingly common problem. The process of vascular injury and repair in this setting shares a number of similarities with, as well as having important differences to, that seen in atherosclerosis. Rates of restenosis and stent thrombosis following coronary intervention have been reported to be as high as 11 and 2 per cent respectively (Mauri *et al.* 2008) although lower rates are generally seen with newer-generation drug-eluting stents (Piccolo *et al.* 2015). Understanding and modifying these adverse responses to vascular injury is crucial if we are to fully realise the potential of catheter-based therapies.

1.2.2.1 Intimal Responses to Mechanical Injury

High-pressure inflation of angioplasty balloons denudes the vessel wall causing crush injury with cell loss and exposure of the sub-endothelial matrix (**Figure 1**). This results in propagation of the coagulation cascade and platelet aggregation with a thin layer of thrombus forming over the injured segment even in the presence of heparin and dual anti-platelet therapy. At 6-12 weeks following stent implantation the thrombus begins to resolve and endothelial cells begin to cover the stented section with full endothelial coverage of the stent taking place by 3 months in most cases (Grewe *et al.* 2000b).

The early thrombus presents an inflammatory milieu with a cellular population made-up largely of activated platelets and neutrophils, later giving way to monocytes and vascular smooth muscle cells (Nakagawa *et al.* 2010; Welt *et al.* 2000). The briskness of this inflammatory response most likely plays a role in determining vascular smooth muscle cell and monocyte behaviour and as such the course of vessel healing and risk of restenosis. Moreover, the magnitude of this response is proportional to the degree of vessel trauma. While a positive correlation between various peri-procedural inflammatory markers and subsequent restenosis has been demonstrated, this has not been a universal finding (Yang *et al.* 2006; Pietersma *et al.* 1995; Morton *et al.* 2005; Xiaohui Zhao *et al.* 2007).

Following the initial acute inflammatory phase of vascular injury, a more chronic cellular profile supervenes, with monocytes, lymphocytes and vascular smooth muscle cells as the main protagonists. It is during this period that neointima formation occurs. Made up largely of vascular smooth muscle, proteoglycan and collagen matrix, the neointima reaches a maximum volume 3-6 months after mechanical injury and is at the core of the restenotic process.

Central to the deposition of extracellular matrix (ECM) and neointimal formation is entry of the vascular smooth muscle cell into the cell cycle, migration into the intima and adoption of a synthetic phenotype (Curcio *et al.* 2011). These synthetic vascular smooth muscle cells are rich in endoplasmic

reticulum, highly proliferative and migratory in contrast with the population within the healthy vessel wall which is rich in contractile elements and has low proliferative and migratory capabilities (Chaabane *et al.* 2014).

1.2.2.2 Medial Responses to Mechanical Injury

Medial injury occurs frequently following endovascular stent implantation with one autopsy study (Farb *et al.* 2002) reporting the presence of fracture of the media in half of arteries studied. In both porcine (Schwartz *et al.* 1992) and human (Farb *et al.* 2002; Nakano *et al.* 2013) studies, the extent of vascular injury during PCI correlates directly with neo-intima formation and re-stenosis. The mechanism behind this is unclear but more extensive injury increases inflammatory cell infiltrate (Farb *et al.* 2002), likely increasing vascular smooth muscle cell activation and ECM deposition.

In summary, balloon angioplasty causes endothelial denudation and an acute inflammatory reaction in the vessel wall, which in turn determines the likelihood of restenosis within the vessel. Acceleration of re-endothelialisation and modification of the inflammatory response may hold the key to preventing restenosis and improving outcomes post-PCI.

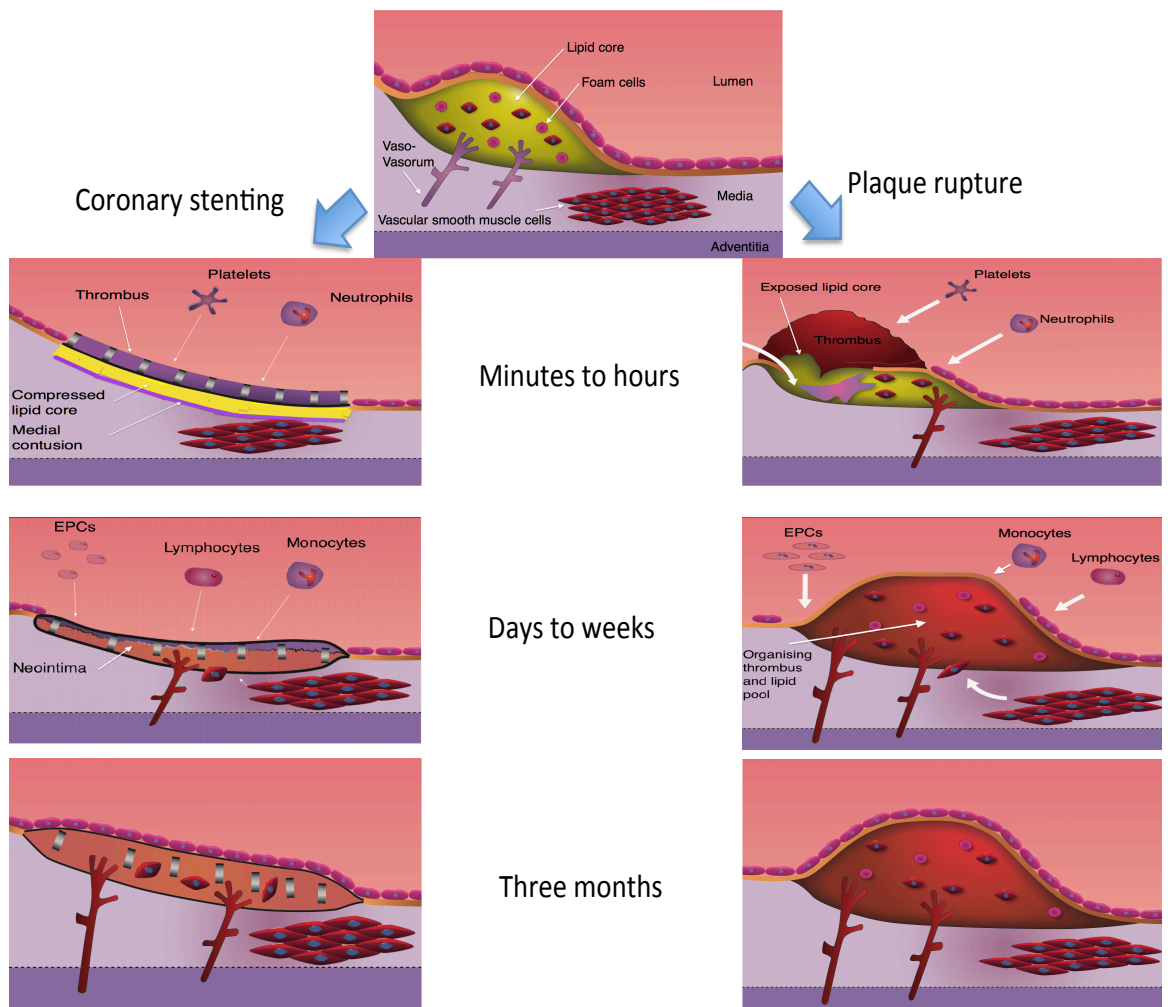


Figure 1: Responses in coronary arteries following plaque rupture and percutaneous coronary intervention

EPCs: endothelial progenitor cells

1.2.3 Vascular grafts

Mechanical injury and its consequences are not limited to percutaneous vascular intervention. Whilst arterial conduits have excellent longevity with 90% patency at ten years, roughly 50% of saphenous vein are occluded by the same time point (Nežić *et al.* 2006).

Exposure of the venous conduit to the higher-pressure arterial circulation causes an increase in radial tissue and surface shear stress (the tangential frictional force of the blood flowing over the endothelium). This leads to endothelial damage followed by arterialisation of the vessels with vascular smooth muscle cell migration into the vessel wall alongside an acute and chronic inflammatory cell influx. The end result is ECM deposition and neointima formation, bearing many similarities to the changes seen following balloon injury (Kaneda *et al.* 2006).

An exaggerated response to injury, early thrombosis (due to operative endothelial injury) and accelerated atherosclerosis account for the three major pathologies leading to graft failure. A recent meta-analysis suggested that atraumatic handling of grafts at the time of surgery improves long term patency rates (Sepehripour *et al.* 2011) emphasizing the importance of the initial injury and inflammatory response in determining outcome in vascular intervention. Pharmacological strategies to modify vein graft longevity have been largely disappointing with aspirin and statins being the only agents proven to inhibit

graft failure (Chesebro *et al.* 1984; Newby 2013). Targeting graft failure with novel cell based therapies has the potential to improve graft patency and clinical outcomes.

1.3 ENDOTHELIAL PROGENITOR CELLS

Following vascular injury, reconstitution of a functional endothelium is a critical step in the recovery of the vessel, allowing the return of vasomotor and thrombomodulatory potential as well as modifying the inflammatory process within the vascular wall. The mechanisms by which this happens, the specific cells involved, and their roles in the reparative process remain controversial.

Early theories proposed that re-endothelialisation occurred from mature endothelium in areas bordering endothelial loss. However, as early as the 1960s, animal studies of vascular grafting (Stump 1963; Shi *et al.* 1994) challenged the traditional paradigm of re-constitution by ingrowth, demonstrating islands of endothelial cells remote from the site of anastomoses. While this early work suggested a circulating endothelial progenitor cell (EPC) in the peripheral blood, it is only following the work by Asahara *et al.* in 1997 (Asahara *et al.* 1997) that this hypothesis has really developed.

Over a decade of research has explored the role of progenitor cells in vascular repair and the paradigm has been refined in many respects.

1.3.1 Circulating endothelial cells: early and late outgrowth

Whilst controversy remains surrounding the phenotypic definition of a true EPC (see discussion below), it is now accepted that early and late-outgrowth endothelial cells, both of which arise from culture of peripheral blood mononuclear cells have divergent roles in vascular repair.

Early outgrowth cells, also known as endothelial cell-colony forming units (EC-CFU), arise from the mononuclear portion of peripheral blood following 5-7 days of culture on fibronectin. They are largely made up of monocytes and lymphocytes (Hill *et al.* 2003) and express high levels of the pan-leukocyte cell marker, CD45. Following culture under angiogenic conditions they express mature endothelial surface markers and can take up acetylated LDL. Additionally Hill and colleagues reported an inverse relationship between EC-CFU concentrations and Framingham risk score (Hill *et al.* 2003).

These observations, alongside the fact that EC-CFU concentrations are increased following acute vascular injury (Hill *et al.* 2003; bonello *et al.* 2006; Marboeuf *et al.* 2007), raised the possibility of a role for these early outgrowth cells in endothelial regeneration. However, it is now clear that these cells have low proliferative potential and are incapable of forming mature endothelial cells (Padfield *et al.* 2013). Therefore, early outgrowth cells likely consist of pro-angiogenic monocytes and lymphocytes supporting vascular repair indirectly through phagocytic and secretory actions, with a separate population directly

responsible for endothelial reconstitution following injury (late-outgrowth endothelial cells).

Late-outgrowth endothelial cells (EOCs), also known as endothelial cell-forming cells (ECFCs), arise following 14-21 days of mononuclear cell culture under angiogenic conditions using collagen rather than fibronectin as an attachment medium. EOCs are capable of forming mature endothelium and are more likely to be responsible for endothelial reconstitution following injury (Yoder *et al.* 2007). EOCs differ from early outgrowth cells in that they have higher proliferative potential and express markers of mature endothelium, such as CD31 and KDR (the extra-cellular domain of the VEGF receptor), rather than haematopoietic markers, and form cobblestone-like sheets similar to mature endothelium in culture.

In keeping with the *in vitro* characteristics of the two populations, early EOCs are unable to incorporate into injured vessels in experimental models of arterial injury (Yoder *et al.* 2007) whereas late EOCs readily incorporate into host vessels and promote angiogenesis and attenuate neointima formation following transplant Barclay *et al.* 2012; Yoder *et al.* 2007; Patel *et al.* 2016; Kang *et al.* 2013; Liu *et al.* 2011). Late outgrowth endothelial cells are therefore an attractive substrate for regenerative therapies.

1.3.2 Defining endothelial progenitor cells

1.3.2.1 Cell surface markers

Whilst it is accepted that EOCs are more likely to be responsible for re-endothelialisation than early outgrowth colonies, and represent the progeny of a true EPC (Yoder 2009), the phenotypic definition of this progenitor cell (from which late-outgrowth cells arise) remains controversial. Attempts to define this population depend upon selecting surface markers of cellular naïveté and an endothelial phenotype prior to culture under angiogenic conditions with the appearance of late-outgrowth cells considered evidence that the progenitor cell is contained within the selected cell populations.

Endothelial progenitor cells were traditionally defined by Asahara as expressing CD34, a stem cell marker and KDR, a receptor for vascular endothelial growth factor found on mature endothelial cells. However, this combination of markers still identifies a heterogeneous population containing cells that give rise to mature endothelium, those with a supportive role in vascular regeneration and those unrelated to the process. Additional markers have been used in an attempt to further define this population (**Table 1**).

CD133 was proposed as an additional marker of cellular naïveté with cells co-positive for CD34, KDR and CD133 considered to be endothelial progenitor cells (Padfield *et al.* 2013; Gill *et al.* 2001). However, recent work has shown that

endothelial cells cannot be raised from a CD133+ population and that CD133-depletion increases the efficiency with which endothelial progenitor cell populations are raised (Peichev *et al.* 2000; Tura *et al.* 2013; Timmermans *et al.* 2007). CD133 likely represents a population of primitive haematopoietic progenitor cells that do not contribute directly to endothelial repair. On a practical level, CD34/KDR/CD133 triple positive cells are very rare in the circulation (Padfield *et al.* 2013; Gill *et al.* 2001; Peichev *et al.* 2000; Timmermans *et al.* 2007) and thus not an attractive candidate for cellular therapy.

Expression of the pan-leukocyte marker CD45 denotes a haematopoietic population that is highly expressed in early outgrowth populations but incapable of raising late-outgrowth endothelial cells (Timmermans *et al.* 2007). CD34 is a pan-stem cell marker that is not specific for endothelial progenitors but late-outgrowth cells likely arise from the CD34 + portion of the peripheral blood mononuclear cells and CD34 enrichment enhances ECFC yield in culture (Tura *et al.* 2013).

Tie-2 (a cell-surface receptor for angiopoietin) expressing monocytes support angiogenesis in pre-clinical models and their concentrations are increased in chronic ischaemic conditions (Patel *et al.* 2013) but their role in formation of mature endothelium is unclear. KDR is highly expressed on mature endothelial cells and late-outgrowth endothelial cells (Yoder 2009). CD146 is expressed widely on immature cells but also seen on mature endothelial cells and it has

been suggested that endothelial progenitor cells may express CD146 with the CD146-negative fraction of mononuclear cells incapable of forming endothelial outgrowth cells in one study (Tura *et al.* 2013)

| Surface marker | Description | Expression | Proposed role of positive cell population in endothelialisation |
|-------------------------------------|--|--|---|
| CD133 | Trans-membrane glycoprotein, function uncertain | Immature haematopoietic cells | Supportive role in vascular regeneration but unlikely to give rise to mature endothelium |
| CD14 | Cell surface marker for bacterial lipopolysaccharide | Monocytes | Supportive role in vascular regeneration but unlikely to give rise to mature endothelium |
| C-Kit (CD117) | Cell surface receptor for stem cell factor | Haematopoietic and multipotent stem cells | Not expressed on mature endothelial cells but progenitor cells may arise from this population |
| CD146 | Trans-membrane glycoprotein, function uncertain | T-lymphocytes, mesenchymal stem cells, endothelial cells and smooth muscle | May contain endothelial progenitor cells giving rise to mature endothelial cells |
| CD45 | Trans-membrane receptor regulating various aspects of cell cycle and differentiation | Haematopoietic cells | Marker of cells with supportive role in vascular regeneration but unlikely to give rise to mature endothelium |
| Tie-2 | Cell-surface marker for angiopoietin | Multi-potent stem cells, leucocytes, monocytes, endothelial cells | Tie-2-positive monocytes likely support angiogenesis, contribution of Tie-2 positive population to formation of mature endothelium unclear |
| CD34 | Adhesion molecule important to cellular migration, other functions uncertain | Multi-potent stem cells | Likely to be expressed on progenitor cell giving rise to mature endothelial cells but heterogeneous population |
| CD31 | Adhesion molecule thought to be important to trans-endothelial migration of inflammatory cells | Platelets, leukocytes, mature endothelial cells | Marker of mature endothelial cells |
| Kinase-domain receptor (KDR) | Surface receptor for vascular endothelial growth factor | Mature endothelium | Expressed on mature endothelium and late-outgrowth endothelial cell populations- Endothelial progenitor cells may reside within this population |

Table 1: cell surface markers proposed for the definition of endothelial progenitor cells.

1.3.2.2 Origin

Alongside the controversy regarding the phenotype of endothelial progenitor cells, there is uncertainty regarding their anatomical origin. The bone marrow was initially thought to be the source of endothelial progenitor cells with studies of non-sex-linked bone marrow recipients showing donor cells incorporated into recipient vessels (Lin *et al.* 2000). However, recent evidence suggests that whilst the bone marrow may supply cells which support angiogenesis (and hence may appear in vessel walls) it does not contain a population from which mature endothelial cells arise (Tura *et al.* 2013; Amini *et al.* 2012; Tsuzuki 2009). This led to suggestions of a population of cells resident within the vasculature from which endothelial progenitor cells arise and a distinct 'vasculogenic zone' has been proposed to exist between the smooth muscle of the media and the adventitia. Consistent with this, progenitor cells have been demonstrated in the walls of both embryonic (Alessandri *et al.* 2001) and mature blood vessels (Ingram *et al.* 2005; Zengin *et al.* 2006).

In summary, it is likely that late, not early, endothelial outgrowth cells are responsible for re-endothelialisation following vascular injury. They arise from the CD34⁺CD45⁻ portion of peripheral blood mononuclear cells and are derived from the vasculature and not bone marrow. The latter assertion is based on observations that late outgrowth endothelial cell yield from mononuclear culture is dramatically increased by CD45 depletion and abolished by positive selection (Timmermans *et al.* 2007). Moreover, neither granulocyte colony-

stimulating factor-mobilised peripheral blood nor bone-marrow aspirate are capable of raising late-outgrowth endothelial cell colonies (Tura *et al.* 2013).

1.3.3 Therapeutic manipulation of the endothelial progenitor cell population

As our understanding of the processes of vascular injury and repair has increased, a number of therapeutic strategies to modify this process have been proposed. These include the modification of progenitor cell populations with pharmacological agents and growth factors, direct stem cell administration and stent-based therapies.

Alongside attempts to directly harness endothelial progenitor cells, various cardio-protective therapies in routine clinical use have been shown to affect EPC biology. The definition of progenitor cells in these studies is variable with some focusing on early outgrowth cells, some defining endothelial progenitors as those exhibiting low-density lipoprotein uptake and lectin binding, and others defining them by cell surface markers (CD34⁺KDR⁺, CD34⁺CD133⁺KDR⁺).

1.3.3.1 Pharmacological Agents

Angiotensin has an inhibitory effect on endothelial progenitor cell function (Sun *et al.* 2013). Angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists reduce restenosis and target vessel revascularisation in

clinical studies (Peters *et al.* 2001; 2005) as well as increasing EPC number in various animal studies (Steinmetz *et al.* 2010; Yu *et al.* 2008). Similarly, mineralocorticoid receptor antagonism has been shown to increase progenitor cell numbers in experimental (Kobayashi *et al.* 2010) and clinical (Jung *et al.* 2012) studies.

Statins, alongside anti-platelet agents, are the most widely used drugs in vascular disease. Among their pleotropic effects, statin use is associated with mobilisation of endothelial progenitor cells and a reduction in senescence (Fadini *et al.* 2010; Paradisi *et al.* 2012; Pirro *et al.* 2009; Baran *et al.* 2012; Walter *et al.* 2004; Spiel *et al.* 2008). Other agents have been shown to influence progenitor cell function but their role is less well established. Calcium channel blockers increase EPC numbers in step with improvements in non-invasive measurements of endothelial function both dependent (Sugiura *et al.* 2008) and independent (De Ciuceis *et al.* 2011) of effect on blood pressure. Beta-blockers increase EPC numbers in animal models of hypertension (Yao *et al.* 2008). Peroxisome proliferator-activated receptor-gamma agonists increase EPC numbers and migratory activity (Pistrosch *et al.* 2005).

1.3.3.2 Growth Factors

Exogenous granulocyte colony-stimulating factor (GCSF) increases the circulating concentrations of putative EPC populations (CD34⁺CD133⁺, CD133⁺KDR⁺, CD34⁺CD133⁺KDR⁺) as well as increasing the numbers of early

outgrowth colonies (Powell *et al.* 2005; Korbliing *et al.* 2006). These observations would perhaps suggest a role for GCSF in promoting vascular repair and indeed there is evidence in mice of accelerated re-endothelialisation with this treatment (Kong 2004; Yoshioka *et al.* 2006). There has however, been concern regarding the increased neo-intima formation and restenosis with GCSF: an effect seen by Yoshioka *et al.* (Yoshioka *et al.* 2006) in mice treated with bare-metal but not drug-eluting stents. This effect was also seen in the MAGIC trial (Kang *et al.* 2004) which randomised patients undergoing bare metal stenting to intracoronary CD34⁺ cell infusion following mobilisation with GCSF alone or standard care. The trial was stopped early due to an unexpectedly high rate of restenosis in the active treatment limbs.

A recent meta-analysis (Ince *et al.* 2008) of post-infarct patients treated with GCSF has shown a neutral effect on re-stenosis regardless of whether bare metal or drug-eluting stents are used. With both negative (Hill *et al.* 2005; Steinwender *et al.* 2006) and positive (Suzuki *et al.* 2006; Meier *et al.* 2009) findings reported, the role for GCSF in coronary artery disease is uncertain but early safety concerns have not been borne out in later trials.

1.3.3.3 Endothelial Progenitor Cell Capture Stents

Whilst the addition of anti-proliferative drugs to vascular scaffolds has significantly improved outcomes following coronary stenting (Piccolo *et al.* 2015), delayed re-endothelialisation and consequent late stent thrombosis

remains a limitation of this technology (Mauri *et al.* 2008). Attempts have been made to harness EPC biology to accelerate re-endothelialisation and protect patients from the risks of stent thrombosis following coronary intervention.

The first iteration of this technology was the Genous R-stent, a bare metal stent coated with anti-CD34 antibodies. Results of clinical trials of this device have been varied. An early small scale trial comparing the stent with a bare metal control (Wojakowski *et al.* 2013) and the eHEALING registry of 4996 patients reported low rates of target-lesion revascularisation (4.4%) and late stent thrombosis (0.3%) at one year follow-up (Silber *et al.* 2011). However, the TRIAS trial of 622 patients (Klomp *et al.* 2011) which randomised patients in a 1:1 ratio to Genous R stent or a drug-eluting stent was terminated early with rates of target vessel failure of 17.4% and 7.0% in these groups respectively. More recently the REMEDEE study (Haude *et al.* 2013) randomised 180 patients to either the (CD34 antibody and sirolimus coated) COMBO™ stent (Orbus-Neich Medical, Fort Lauderdale, Florida) or paclitaxel DES and showed no difference in restenosis at 12 months with subsequent registry data of 1000 patients treated with combo stent reporting equivalence with second-generation DES in terms of target-vessel failure (Woudstra *et al.* 2016).

Whilst CD34 coated stents accelerate re-endothelialisation, the cell population recruited to the site of injury is heterogeneous and is likely to include vascular smooth muscle, haematopoietic and pro-inflammatory cells in addition to endothelial progenitor cells (Takabatake *et al.* 2014). This may, in part, explain

the excess of restenosis observed in clinical trials. Attempts have been made to address this by both using different progenitor cell-capture coatings and combining antibody coating with anti-proliferative agents. At least in preclinical porcine models, the use of more endothelial-specific coatings such as vascular endothelial cadherin (Lim *et al.* 2011) and vascular endothelial growth (Takabatake *et al.* 2014) factor have shown favourable results compared to CD34-coated stents.

In addition to alternative antibodies, polymer microarray technology has identified novel biosynthetic polymers which promote endothelial cell attachment and may promote re-endothelialisation whilst minimising platelet adherence (Pernagallo *et al.* 2012).

Stent-based manipulation of EPC biology is an appealing prospect but the issue of non-specific cellular recruitment will have to be addressed and optimal results will likely require the combination of antibodies, polymers and anti-proliferative agents.

1.3.4 Cell therapies for acute and chronic ischaemic disease

In cardiovascular medicine cell therapy trials have largely addressed myocardial regeneration. Although increased left ventricular ejection fraction has been the primary outcome in many regenerative medicine trials and myocardial regeneration is a distinct process from angiogenesis, many

investigators have also examined the effects of cell therapy on myocardial perfusion with some looking more directly at revascularisation with stem cell therapy (**Table 2**). Encouraging though these early trials have been, they have suffered from small numbers and often lacked appropriate controls.

| Author (year) | Cell therapy product | Delivery | Control | Population (n) | Outcome |
|-------------------------------|--|-------------------------|---------|------------------------------------|--|
| Jimenez-Quevedo (2014) | GCSF-mobilised CD133 cells | Intra-myocardial | Yes | Refractory angina (28) | ↓ Angina frequency ↑ Perfusion ↑ Exercise tolerance |
| Losordo (2012) | GCSF-mobilised PB CD34+ cells | Intra-myocardial | Yes | Refractory angina (167) | ↓ Angina frequency ↑ Walk time ↑ Perfusion → ↑ |
| Losordo (2012) | GCSF-mobilised PB CD34+ cells | Intra-muscular | yes | Critical limb ischaemia (28) | ↓ Amputation ↓ Rest pain ↑ Walk time |
| Lasala (2011) | Marrow-derived MSC population plus MNC fraction from BM aspirate | Intra-coronary | No | Stable CCS III/ IV angina (10) | ↑ Perfusion (single photon emission tomography) ↓ Angina frequency |
| Murphy (2011) | BM aspirate. Analysis of population by CD133, CD34, KDR positivity done but not reported | Intra-muscular | No | Critical limb ischaemia (CLI) (29) | ↑ Amputation-free survival ↑ Perfusion (positron emission tomography) |
| Tuma (2011) | BM-derived MNCs and CD34+ fraction | Coronary sinus infusion | No | Refractory angina (14) | ↓ Angina frequency ↑ Myocardial perfusion (single photon emission tomography) ↑ Walk time |
| Lasala (2010) | BM-derived cells Mononuclear cell populations plus 'progenitor population' (CD34+CD133+CD144+) | Intra-muscular | No | Chronic lower limb ischaemia (11) | ↑ Limb perfusion (digital subtraction angiography) ↑ Quality of life score ↑ Ankle-brachial pressure index |
| Boyle (2006) | GCSF-mobilised CD34+ cells | Intra-coronary | No | Stable coronary artery disease (5) | ↑ Collateral flow on repeat angiography ↓ Angina frequency |
| Erbs (2005) | GCSF-mobilised 'CPCs' | Intra-coronary | Yes | Coronary artery disease (26) | ↑ Coronary flow-reserve ↑ Ejection fraction → In-stent restenosis |

Table 2: Cell therapy trials for chronic ischaemic disorders

A recent Cochrane review of stem cell therapies in chronic ischaemic heart disease and congestive cardiac failure (Fisher *et al.* 2016) concluded that whilst there was evidence of mortality benefits with these therapies, the improvements in cardiac function are modest and overall the quality of the evidence was low. Experience of cell therapy for acute myocardial infarction has been similarly disappointing with a 2015 Cochrane review (Fisher *et al.* 2015) concluding that there is no mortality or quality of life benefit with these therapies and again that the quality of evidence is generally poor.

It was hoped that larger-scale Phase 3 clinical trials would address some of these shortcomings but unfortunately such trials, the ATHENA (Henry *et al.* 2016) and RENEW (Povsic *et al.* 2013) studies were recently terminated early for an excess of adverse events and slow-enrolment respectively. Another study, the CHART-1 trial (Bartunek *et al.* 2017), although not raising any safety concerns, was negative. There are however two large-scale randomised-controlled trials examining cellular therapy for heart failure: BAM1 (NCT01569178) and DREAM-HF (NCT02032004) and which will provide crucial information about the efficacy of these treatments.

A major issue with cell therapy trials to date is that they have largely utilised a non-specific population of mononuclear cells, derived either from direct bone-marrow extraction or from peripheral blood following granulocyte colony-stimulating factor-mobilisation, with CD34 positive selection being the only

refinement. As a result, the cell suspensions tested have included heterogeneous populations which may have led to the dilution of cells with true therapeutic potential and attenuation of their beneficial effects. Better results may be seen with the use of culture-modified populations which would allow administration of well-characterised phenotypically homogeneous populations.

Successful application of this approach will require careful selection of the cellular population. With their robust proliferative potential (Yoder 2009) and proven ability to incorporate into host vessels, and contribute to vasculogenesis in experimental models (Barclay *et al.* 2012; Dubois *et al.* 2010), late-outgrowth endothelial cells may represent such a substrate for cellular therapy.

As well as the possibility of tissue regeneration in ischaemic disease, endothelial outgrowth cells may also have therapeutic potential in the tissue engineering of vascular conduits. At present, large calibre vessels can generally be replaced or bypassed with conduits made from synthetic polymers. The absence of an intact functional endothelial layer at implantation increases the risk of conduit failure due to thrombotic occlusion, infection or rejection. In smaller vessels (<6mm) synthetic conduits are prone to thrombosis and autologous vessels (either venous or arterial) are still preferred.

Tissue engineering using decellularised scaffolds either seeded with cells of the vessel wall or designed to capture these cells in situ, offers a potential solution to these problems. This approach has been shown to be both feasible and

effective in the preclinical setting (Muylaert *et al.* 2014) and in early clinical work in a series of 10 haemodialysis patients with limited vascular access option (McAllister *et al.* 2009) who received grafts constructed from autologous fibroblast extra-cellular matrix and seeded with culture-expanded endothelial cells. More recently decellularised human fibroblast-derived ECM conduits have raised the possibility of an 'off the shelf' bio-engineered conduit (Lawson *et al.* 2016).

Although in its infancy in the clinical arena, tissue engineering, either in the form of pre-formed multi-layered vessels coated with an endothelial cell product or resorbable scaffolds which promote in-situ chemotaxis and seeding of endogenous endothelial cells, has the potential to revolutionise conduit choice and vascular grafting in man.

1.4 THE RADIAL ARTERY AS A TOOL FOR STUDYING VASCULAR INJURY AND REPAIR

Full realisation of the therapeutic potential of endothelial progenitor cells will require further characterisation of their behaviour as well as their modifying effects on the processes of vascular injury and repair. Whilst EOCs are well-characterised *in vitro* (Yoder *et al.* 2007) and their regenerative capabilities are well-demonstrated in animal models (Barclay *et al.* 2012; Minami *et al.* 2015; Schwarz *et al.* 2012), detailed human *in vivo* data is lacking.

Previous work has characterised EPC responses to vascular injury in the context of coronary angiography (Padfield *et al.* 2013; Padfield *et al.* 2014; Gao *et al.* 2015; Thomas *et al.* 2008), this work has however lacked detailed characterisation of the injury sustained and an assessment of the modifying effects of EPC/EOC biology on vessel recovery.

The radial artery in the context of transradial cardiac catheterisation represents an opportunity to bridge this gap in the understanding of EPC biology. Widely accepted to be superior to the femoral approach for diagnostic angiography and therapeutic percutaneous coronary intervention (Cruden *et al.* 2007; Jolly *et al.* 2011), it has become the access route of choice in the majority of UK centres ('BCIS National Audit of Percutaneous Coronary Interventions' 2016). Despite the clear advantages of this route, radial artery cannulation commonly causes structural injury which has been demonstrated in two moderate-sized intra-vascular imaging-based studies (Yonetsu *et al.* 2010; Di Vito *et al.* 2013) as well as with ultrasound (Costa *et al.* 2016). Whilst this injury is rarely of clinical consequence, functional impairment of the radial artery following catheterisation is common. Several studies have employed flow-mediated dilatation (FMD) to demonstrate functional impairment of the radial artery following catheterisation (Yan *et al.* 2010; Dawson *et al.* 2010; 2012; Burstein *et al.* 2007) with variable recovery reported. Given the dependence of FMD on an intact endothelium, the impairment reported in these studies likely reflects endothelial denudation with any recovery seen reflecting re-endothelialisation.

Thus the radial artery in the context of cardiac catheterisation affords the opportunity to study endothelial progenitor cell biology and relate this to intravascular imaging-defined arterial injury and vasomotor function.

1.5 TRACKING CELLS IN VIVO

As well as the beneficial effects on organ structure and function following administration of a cellular therapy, it is essential to demonstrate localisation of the cells to the site of action to provide bio-plausibility- especially important given the logistical and financial challenges of conducting adequately-powered clinical trials of cell therapies. The ideal method for cell tracking *in vivo* would be non-invasive and allow high spatial resolution and longitudinal definition of cell fate without adversely affecting cell viability or function.

1.5.1 Labelling cells for tracking studies

Labelling techniques are generally described as direct (where there is incorporation of the label into the administered cells), receptor-based or reporter-gene based. Reporter gene-based techniques are favoured in pre-clinical work due to the fact that they allow longitudinal cell tracking and reflect the activity of viable cells given their reliance on RNA transcription. Concerns over genetic manipulation of administered cells and poor tissue penetration of fluorescent or bioluminescent probes used limit their use in man. Direct-labelling techniques are the most widely used in clinical studies to date with

positron-emission tomography (PET) predominating. As well as the fact that there are generally fewer safety concerns allowing more rapid clinical translation, PET has excellent tissue penetration, sensitivity and anatomic definition when combined with computed tomography (CT) (Nguyen *et al.* 2014).

1.5.2 Direct labelling with radioisotopes for cell tracking

Direct labelling of cells with radioisotopes is employed in single positron emission tomography (SPECT) and positron emission tomography, both of which can be combined with computerised tomography (SPECT-CT or PET-CT respectively) to provide anatomical information for tracking studies.

Most commonly using ^{111}In -oxine (^{111}In -oxine) or ^{99}Tc -hexamethylpropylenamine oxime (^{99}Tc -HMPAO), SPECT scanning is predominantly used in cancer imaging or in detection of occult infection, but has been used in cardiovascular cell tracking studies (Blocklet *et al.* 2006; Schachinger *et al.* 2008; Kurpisz *et al.* 2007; Caveliers *et al.* 2007) While SPECT tracers have a longer half-life than PET tracers, spatial resolution of SPECT CT is inferior to PET CT (Chan *et al.* 2012): a major drawback in vascular imaging.

^{18}F -Fluorodeoxyglucose (^{18}F -FDG) is the most commonly used PET tracer (Chan *et al.* 2012) and is discussed below. Alternative PET tracers have been used for pre-clinical cell labelling work, notably ^{18}F -fluorothymidine (^{18}F -FLT) and

Hexadecyl-4-[^{18}F]-fluorobenzoate (^{18}F -HFB). ^{18}F -FLT is a nucleotide analogue and accumulates in actively dividing cells, it has been used clinically in cancer imaging (Szysko *et al.* 2016) and has been shown to be effective in pre-clinical trials of cell labelling (MacAskill *et al.* 2017). ^{18}F -HFB is a lipophilic molecule which is taken up by the cell membrane and whilst effective cell labelling has been demonstrated in pre-clinical work, there are concerns over acute toxic effects on cells (Zhang *et al.* 2012) and its use remains restricted to pre-clinical work.

1.5.3 ^{18}F -Fluorodeoxyglucose

^{18}F -Fluorodeoxyglucose is a glucose analogue with a hydroxyl group substituted for the radioactive isotope, ^{18}F -fluorine, and is the most widely used radioisotope in cell tracking studies (Rosado-de-Castro *et al.* 2014). It is taken up by metabolically active cells through insulin dependent and independent transport mechanisms. Following uptake, it is phosphorylated but cannot progress beyond this initial step of glycolysis and is trapped in the cell by a concentration gradient and accumulates in proportion to glycolytic activity in metabolically active cells.

There are several limitations of ^{18}F -FDG as a tracer for cell labelling with a relatively short half-life (110 minutes) and significant loss of the tracer over the first two hours post-labelling (Doyle *et al.* 2007; Zhang *et al.* 2012) precluding long-term tracking of cell fate. However, it has been extensively and successfully

utilized in cardiovascular cell tracking studies (Doyle *et al.* 2007; Hofmann *et al.* 2005; Dubois *et al.* 2010; Blocklet *et al.* 2006) and has a well-established safety profile. There is expertise in its manufacture and use in Edinburgh, and when combined with PET-CT, ^{18}F -FDG provides excellent spatial resolution.

1.6 SUMMARY

Endothelial progenitor cells play an important but incompletely defined role in vascular repair and manipulation of EPC populations may have a future role as a therapy for cardiac regeneration. The radial artery in the context of transradial cardiac catheterisation offers the opportunity to study the dynamics of progenitor cell populations and their influence on functional vessel recovery following defined vascular injury a readily available, accessible and controlled manner. Additionally, the combination of a cell therapy grade manufacturing process for late outgrowth endothelial cells and radio-nucleotide labelling would allow tracking studies with these cells and further mechanistic insights into the processes of vascular repair.

1.7 AIMS AND HYPOTHESES

Aims

1. To establish the feasibility of using the radial artery to study vascular injury and repair using the technique of flow-mediated dilatation.
2. To define the structural and functional consequences to the radial artery of transradial cardiac catheterisation using optical coherence tomography (OCT) and FMD respectively alongside the cellular profiles accompanying this and the modifying effect of these on vessel recovery.
3. To develop a good manufacturing practice-compliant culture process for production of late-outgrowth endothelial cells from peripheral blood.
4. To develop a labelling protocol using ^{18}F -FDG with a view to a cell-tracking study in man using positron-emission and computed tomography.

The following hypotheses will be addressed:

1. Transradial cardiac catheterisation causes a reversible decline in radial vasomotor function which can be studied longitudinally using flow-mediated dilatation (Chapter 3).
2. Vascular injury sustained at the time of radial catheterisation causes a mobilisation of endothelial progenitor cells (Chapter 4).
3. Endothelial progenitor cell concentrations and function influence vessel recovery following injury (Chapter 5).
4. Late-outgrowth endothelial cells can be isolated and expanded in a GMP-compliant manner which will allow human administration (Chapter 6).
5. Late-outgrowth endothelial cells can be radiolabelled using ^{18}F -FDG without compromising their viability or functionality (Chapter 7).

Chapter 2

Materials and Methods

2.1 ETHICAL AND REGULATORY CONSIDERATIONS

All studies were performed with approval of the local research ethics committee and conducted in accordance with the Declaration of Helsinki (2013). Informed consent was obtained from all participants prior to study enrolment. The longitudinal study of radial artery function (Chapters 4 and 5) and translational work to develop a GMP-compliant endothelial outgrowth cell culture process (Chapter 6) were registered on the public website www.clinicaltrials.gov (NCT02147119 and NCT02975518 respectively).

2.2 STUDY POPULATIONS

2.2.1 Healthy volunteers

Healthy volunteers were recruited by advert from staff and students at the University of Edinburgh. All volunteers were free from significant chronic medical conditions or intercurrent illness and took no regular medications. Pregnant women and current smokers were excluded.

2.2.2 Patients

All patients were recruited from stable populations awaiting outpatient coronary angiography for investigation of known or suspected coronary artery disease at the Royal Infirmary of Edinburgh. Patients with an absent radial pulse, those experiencing a recent acute coronary syndrome or recent cardiac catheterisation (<3 months), severe valvular heart disease, renal impairment

(estimated glomerular filtration rate <30mL/min), contrast allergy, pregnancy, inability to give informed consent and those aged below 18 or above 85 years were excluded. All patients donating blood to be used in the clean room environment were tested for blood-borne virus status (HIV 1 and 2, Human T-lymphotrophic virus-1, Hepatitis B and C) prior to venesection.

For assessment of radial artery function, study visits were carried out in a quiet, temperature-controlled room at the same time of day to minimise biological variation. Subjects were asked to avoid food for four hours prior study visits and caffeine, smoking and vasoactive medications for twenty-four hours.

2.3 VENESECTION

For EOC culture and EPC enumeration (Chapters 4 and 5), 100 mL whole blood was collected from a large peripheral vein into two 50- mL tubes, each containing 4 mL of 3.8% Sodium Citrate as an anticoagulant.

For EOC GMP culture protocol development and validation, (Chapter 6) 250 mL of blood was collected from a large peripheral vein into sealed collection bags (Macropharma Phillipines) containing sodium citrate. Blood to be used in clean room culture processes was collected in the clinical apheresis unit at the Royal Infirmary of Edinburgh.

2.4 FLOW AND NITRATE-MEDIATED DILATATION

2.4.1 Background

Flow and nitrate-mediated dilatation are well established methods for assessing endothelium-dependent and independent vasomotion respectively. A hyperaemic stimulus is achieved by inflation then deflation of a supra-systolic cuff, the resultant increased shear stress causes nitric oxide release from healthy endothelium and relaxation of vascular smooth muscle and vasodilatation (FMD). Endothelial dysfunction causes attenuation of this response which correlates with cardiovascular risk in large population studies (Celermajer *et al.* 1989) and coronary dysfunction and disease at invasive angiography. Although the technique is usually carried out using the brachial artery, we studied the radial artery given our requirement to study local injury post catheterisation. The reproducibility of brachial FMD is variable in the literature with coefficients of variation reported to be as low as 2% but often higher (Sorensen *et al.* 1995; Harris *et al.* 2007; Charakida *et al.* 2013) and we sought to define the reproducibility of radial FMD in our own work which is outlined in chapter 3.

2.4.2 Image acquisition (B-mode ultrasound)

Flow and nitrate-mediated dilatation (FMD and NMD respectively) were carried out according to international guidelines. The radial artery was imaged

longitudinally 5 cm proximal to the radial styloid with a 12-15 MHz linear-array ultrasound transducer (CX50 Phillips, Netherlands) held in place by a stereotactic clamp. Baseline imaging was performed over 60 seconds. A supra-systolic cuff was then inflated to 220 mmHg for 5 minutes immediately distal to the antecubital fossa. Following release of the cuff, the artery was imaged continuously for five minutes (FMD). After a further 15 minutes of rest, the artery was again imaged for 60 seconds. Subjects were then given 25 µg of sublingual nitrate and the radial artery imaged for continuously for a further 5 minutes (NMD).

Image acquisition was ECG-gated with arterial diameter captured during end-diastole (R-wave triggered). The artery was initially identified using colour-flow mapping. The probe position that gave the largest arterial diameter and clearest definition of the anterior vessel wall was chosen to minimise under-estimation of the lumen. The focus position of the probe was set to the anterior vessel wall.

2.4.3 Radial artery image analysis (B-mode ultrasound)

Images were analysed offline using proprietary software (Brachial Analyser, Vascular Tools, Medical Imaging Applications, USA). Baseline and peak diameters were measured in millimetres with measurements averaged across sixty frames and ten frames for baseline and peak values respectively. Flow-mediated dilatation and NMD were expressed as percentage change in diameter from baseline.

2.5 TRANSRADIAL CARDIAC CATHETERISATION

Coronary angiography was performed via the right radial artery in all cases. Briefly, following local anaesthesia with 1% lidocaine, the radial artery was punctured using the Seldinger technique and a 6-French arterial sheath (Check-Flo™ 13cm, Cook medical, USA) was inserted. Heparin was administered when access to the aortic root was confirmed (5000 units bolus with additional boluses at operator's discretion if follow-on percutaneous coronary intervention (PCI) was undertaken). Arterial sheaths were removed at the end of the procedure with haemostasis achieved using a TR band™ (Terumo, Japan). In a subset of patients (n=10), radial sheaths were examined post-procedure for the presence of endothelial cells as described below.

2.6 RADIAL ARTERY OPTICAL COHERENCE TOMOGRAPHY

2.6.1 Background

Optical coherence tomography (OCT) is an imaging technique utilising near infra-red light and is used in several medical applications, most widely in retinal imaging in ophthalmology. It was first used for human intra-vascular imaging in 2008 (Kume *et al.* 2008). A catheter containing a rotating light source and detector is passed into the coronary arteries over a guidewire and examination of the light backscatter allows construction of 2D images with axial resolution of 10-20 µm (Kume *et al.* 2008). This technique is used clinically to guide stent

implantation and elucidate mechanisms of stent failure and has also been used previously to image the radial artery following catheterisation (Yonetsu *et al.* 2010).

2.6.2 Image acquisition

Optical coherence tomography of the radial artery was performed at the time of diagnostic angiography prior to sheath insertion and after sheath withdrawal. Briefly, the arterial sheath was initially inserted 2 cm into the vessel, a coronary guide wire (Balance Middleweight, Abbot Vascular, USA) was then passed to the brachial artery under fluoroscopic guidance over which an OCT catheter (Fastview™ Terumo, Japan) was advanced with position monitored using live imaging. A pullback was performed of the 15 cm of artery proximal to sheath insertion. The sheath was then inserted to its full length and the angiogram performed as usual. At the end of the case the sheath was withdrawn to its original 2 cm point and the artery imaged again as above. All patients received 200 µg of intra-arterial glyceryl trinitrate prior to each imaging run.

2.6.3 Image analysis

Vessel dimensions were assessed at 1-cm intervals along the imaging run with three consecutive frames analysed at each location. Areas of the internal elastic lamina (IEL), external elastic lamina (EEL) and radial artery lumen were measured using proprietary software (Lunawave, Terumo, Japan) and manual

tracing. Mean intimal area (average internal elastic lamina area minus average luminal area) was calculated along the length of the vessel. Structural injury was assessed qualitatively with each imaging run analysed on a frame-by-frame basis. Only lesions present on at least three consecutive frames were adjudicated as injuries. Where identified, injury was classified as an intimal tear (if a flap was visible but did not extend beyond the intima) or as a medial dissection (if the injury extended into the smooth muscle layer). This convention has previously been employed for study of mechanical arterial trauma (Yonetsu *et al.* 2010; Di Vito *et al.* 2013).

2.7 FLOW CYTOMETRY FOR ENDOTHELIAL PROGENITOR CELL POPULATIONS

Flow cytometry is a method most commonly used for defining the markers expressed on the cell surface. It incorporates antibodies to cell-surface markers that are conjugated with fluorophores- chemical compounds which re-emit light when excited by incident light of a specific wavelength. Cell suspensions are incubated with conjugated antibodies and then washed to remove non-adherent antibody. They are then examined in a flow cytometer which suspends cells in a single-cell column and passes them through lasers of various wavelengths with each antibody/fluorophore combination assigned a 'channel' based on the wavelength at which they are excited the wavelength of the light emitted following excitation. Software then plots each cell as a point on a scatter plot

based on its fluorescent intensity and from this, cell-surface marker expression can be deduced.

As with most flow cytometry panels, the EPC panel employed in our laboratory consists of several antibodies used together. This presents a potential source of error if the emission spectra (the wavelength of light emitted when the fluorophore is excited by the laser in the flow cytometer) overlap and this can cause false positives with fluorescence fluorophore/ antibody combination attributed to another. To avoid this, compensation is performed. This involves running positive samples (either stained cells or beads with adherent fluorophore) and examining the spill-over of fluorescence into other channels (which should have no fluorescence in the absence of the appropriate fluorophore). A correction is then applied using the flow cytometer's software package.

Fresh blood was stained with panels of pre-conjugated anti-human monoclonal antibodies to quantify putative EPC populations: CD45-V450 (BD horizon), CD34-APC/Cy7 (Biolegend, USA), KDR-PE (BD Biosciences, USA), CD133-APC (Miltenyl Biotec, Germany), KDR-PE (BD Biosciences, USA).

Unstained samples were used as negative controls and compensation was performed using commercially available beads (Miltenyi Biotec, UK). Samples

were processed using a 4-laser flow cytometer (LSR-Fortessa II, BD, USA) with 80,000 events captured.

Offline analysis was carried-out using Flowjo™ (Treestar, USA). Debris was first excluded and the leukocyte population identified from its typical forward/ side-scatter profile. Expression of each surface marker was then determined individually and co-expression was determined using Boolean principles. Previous unpublished work by our laboratory using the same protocol has recorded intra-assay co-efficient of variation of 0.1% for CD45, 9% for CD34, 6% for CD133 and 8% for KDR.

2.8 QUANTIFICATION OF ENDOTHELIAL CELLS FROM ARTERIAL SHEATHS

The lumen of the sheath was flushed to remove blood and the sheaths were placed in phosphate-buffered saline and centrifuged at 5000 rpm for 10 minutes (Eppendorf 5702, Eppendorf, Germany). Cells obtained were resuspended in PBS and incubated with the endothelial cell antigen CD31-PE (BD Biosciences, USA), platelet marker CD42a-FITC (BD Biosciences, USA) and haematopoietic cell antigen CD45-APC (BD Biosciences, USA) and 40,000 events were acquired using a 4-laser flow cytometer (LSR-Fortessa II, BD, USA). Gating strategies are included as a figure in **Chapter 4**.

2.9 LATE-OUTGROWTH ENDOTHELIAL CELL ISOLATION

Late-outgrowth endothelial cell isolation is well described (Yoder *et al.* 2007) and involves culture of unmobilised peripheral blood mononuclear cells under angiogenic conditions. These cells arise as clusters (or colonies) following 7-14 days of culture and proliferate to form a cobblestone monolayer similar to mature endothelium.

Mononuclear cells were isolated from peripheral blood using gradient density centrifugation (Eppendorf 5702, Eppendorf, Germany) with Ficoll Paque PLUS (GE healthcare, Sweden). Following resuspension in endothelial growth medium (EBM II Lonza, Switzerland) with 10% Hyclone foetal bovine serum (Lonza, Switzerland) 1×10^6 mononuclear cells were seeded onto type-1 rat's tail collagen-coated 6-well plates (BD Biosciences, USA). Cells were incubated at 37°C, 5% CO₂, 95% relative humidity. Medium was changed twice per week. Late-outgrowth colonies were counted as they emerged and cells were harvested using trypsin-EDTA for further analysis at 3 - 4 weeks.

2.10 CELL LABELLING

2.10.1 Fluorodeoxyglucose

¹⁸F-Fluorodeoxyglucose is a glucose analogue with a hydroxyl group substituted for the radioactive isotope fluorine and is the most widely used

radioisotope in cell tracking studies (Rosado-de-Castro *et al.* 2014). It is taken up by metabolically active cells through insulin dependent and independent transport mechanisms and trapped intra-cellularly by phosphorylation. It is manufactured onsite at the Clinical Research and Imaging Centre at the Royal Infirmary of Edinburgh and was provided with an initial activity of 500 Megabecquerels (MBq)/mL.

2.10.2 ^{18}F -FDG incorporation

Confluent flasks of EOCs were trypsinised, cells were then counted and resuspended in saline supplemented with 0.5% human albumin solution (Lonza, Switzerland). Cells were then incubated with ^{18}F -FDG for 30 minutes at 37°C and agitated at 300 Hz using an automated mixer (Thermomixer®, Eppendorf, Germany). Unincorporated tracer was removed with a three-step washing protocol using saline and 300 g, 5-minute spins. Leak was determined at 60 and 120 minutes by sampling the supernatant following centrifugation of the cell suspension. Samples of the stock ^{18}F -FDG solution, washing supernatant and the final resuspended cellular pellet were read in a gamma counter (1470 Wizard, PerkinElmer lifesciences, USA). Inter-sample co-efficient of variation for FDG incorporation was 19%. All counts were decay-corrected to the time of incubation and expressed in Megabecquerels (MBq).

Low volume, small cell number experiments were first conducted to construct dose-response curves for ^{18}F -FDG incorporation. Volumes and cell numbers

were then scaled up to those to be used in human administration Volumes and cell numbers are stated in all figures in **Chapter 7**.

2.10.3 Assessment of ^{18}F -FDG leakage following labelling

Labelled cells were suspended in endothelial basal media at 37°C. At one hour, the suspension was centrifuged and the activity of the supernatant measured. The cell pellet was then re-suspended and incubated at 37°C for a further hour before centrifugation and measurement of the supernatant to give the leakage over two hours.

2.11 ASSAYS FOR PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF LATE-OUTGROWTH ENDOTHELIAL CELLS

2.11.1 Endothelial cell markers and viability

EOCs were extensively characterised in terms of their cell surface markers at the point of release (culture day 32). Cells were incubated with CD45-APC (BD Biosciences, US), CD31-FITC DRAQ7 (BD Biosciences, US), DRAQ7-APC/Cy7 (Biostatus, UK) and analysed on a BD FACSCANTO II flow cytometer (BD Biosciences, US). High expression of CD31 (>90%) and low CD45 expression (<10%) alongside viability >50% were pre-requisites for product release. Extended phenotyping was carried out (CD34, CD144, CD146, KDR) for information.

2.11.2 Sterility

Sterility of the cell product was confirmed by inoculation of BactALERT™ (Biomérieux, France) aerobic and anaerobic culture bottles with spent media at each media change and with the cell product on the day of release. BactALERT™ is an automated system which uses a colorimetric sensor to detect the CO₂ produced by bacteria or fungi growing in a supplemented broth inoculated with either spent media or body fluids.

2.11.3 Zombie green dye viability assessment

Zombie Green™ dye is a fluorescent dye which is non-permeant to viable cells but permeant to cells with compromised membranes and thus can be used to determine cell viability. Following suspension in PBS, cells were incubated with Zombie Green™ dye (Biolegend, USA) (1:1000 solution for 20 minutes). Samples were fixed and analysed using a 4-laser flow cytometer (LSR Fortessa BD biosciences, USA). Analysis was performed offline using proprietary software (Flowjo, treestar, USA). Co-efficient of variation for the assay was 7.7%.

2.11.4 Proliferative activity

Following labelling and washing cells were re-suspended in endothelial growth medium (EBM2, Lonza, Switzerland) and counted. 1×10^5 cells were plated to

rat's tail collagen-coated 6-well plates (BD, USA) and EBM-2 was added. Non-adherent cells were removed at 24 hours and medium was thereafter changed twice per week. Cells were counted using a haemocytometer and manual counting at 24 hours, 72 hours and 7 days.

2.11.5 Cell cycle analysis

Cell cycle analysis uses the DNA content of the cells to determine the proportion of cells at each stage of mitosis within a population. DAPI is a fluorescent dye which binds to DNA with the fluorescence of the cell directly proportional to DNA content which varies with the cell's position in the cell cycle. There are typically three populations seen on a cell cycle frequency histogram which correspond to different stages of the cell cycle: G₀/G₁, the largest population, represents the initial stage where cells grow and DNA is replicated, S represents the cellular population undergoing protein synthesis and G₂ represents a cellular population immediately prior to mitosis when the DNA content of the cell is highest. There is an important check-point in the cell cycle following the G₂ phase and genetic damage caused by radiation typically causes an arrest of cell cycle at this point after the synthetic and growth phases prior to entering mitosis, termed G₂M accumulation.

Collagen coated T25 flasks were plated with 1×10^6 labelled cells and cultured as above for cell cycle analysis. At 48 hours, cells were trypsinised (TrypLE™, Thermo Fisher, USA), DAPI prep™ (Sony Biotech, USA) was added and 20,000

events were acquired on a 4-laser flow cytometer (LSR Fortessa, BD Biosciences, USA). Analysis was performed offline using proprietary software (Flowjo, treestar, USA). Inter-sample co-efficient of variation was 7.3% for the largest (G_0/G_1) population and 46 and 41% for S and G_2M populations respectively.

2.11.6 EOC adhesion

EOCs were suspended in endothelial growth medium as above. 4×10^4 cells were added to 6-well plates coated with Rat's tail collagen with 1 mL of EBM2 (Lonza, Switzerland) and incubated at 37°C for 30 minutes. Plates were then washed three times and cells were fixed using formalin. Plates were imaged using inverted light-source microscopy (Zeiss Observer, Carl Zeiss, Germany) and adherent cells expressed as percentage of the original cell number. Inter-sample co-efficient of variation was 21%.

2.13 Statistical analysis

Bland-Altman plots were used to examine the reproducibility of flow and nitrate-mediated dilatation. Continuous variables were compared using two-sided student's t-tests. Continuous data with multiple time points or datasets were compared using analysis of variance (ANOVA) with multiple comparisons and Tukey or Bonferroni post-tests. Categorical data was analysed using Chi-squared tests. Correlation was performed using person and spearman tests. All

analysis was carried out using Statistical package for the Social Sciences (SPSS, IBM, USA) or GraphPad Prism (Graphpad software inc, USA) where appropriate. Statistical significance was taken as a two-sided p value of <0.05 .

Chapter 3

Reproducibility of radial artery flow-mediated dilatation and feasibility as a model of vascular injury

Data from this chapter is published in *Open Heart*:

Mitchell AJ, Mills NL, Newby DE, Cruden NL 'Radial artery vasomotor function following transradial cardiac catheterisation' *Open Heart* (2016);**3**:e000443

3.1 SUMMARY

Flow and nitrate-mediated dilatation (FMD and NMD) are well established non-invasive methods of assessing vascular function. However, the majority of this work has focused on the brachial artery and whilst some investigators have studied FMD and NMD of the radial artery following transradial cardiac catheterisation, the results have been conflicting and reproducibility of this technique is not well described.

The aim of this study was to determine the reproducibility of flow- (FMD) and nitrate- (NMD) mediated dilatation in the assessment of radial artery vasomotor function. Additionally, it sought to determine whether impairment of vascular function could be detected in a small cohort of patients following transradial catheterisation and thus whether the radial artery represented a feasible model of mechanical vascular injury.

Radial artery FMD and NMD were examined in 20 volunteers and 20 patients on four occasions (two visits at least 24 h apart, with two assessments at each visit). In a further 10 patients, radial artery FMD was assessed in the catheterised arm prior to, at 24 h and three months following cardiac catheterisation.

There were no differences in baseline radial artery diameter (2.7 ± 0.4 mm *versus* 2.7 ± 0.4 mm), FMD (13.4 ± 6.4 *versus* $12.89 \pm 5.5\%$) or NMD ($13.6 \pm 3.8\%$ *versus*

10.1±4.3%) between healthy volunteers and patients ($p>0.05$ for all comparisons). Mean differences for within and between day FMD was 2.53% (95% confidence intervals, -15.5 to 20.5%) and -4.3% (-18.3 to 9.7%) in patients. Compared to baseline, radial artery FMD was impaired at 24 h (8.7±4.1% *versus* 3.9±2.9%, $p=0.015$) but not 3 months (8.7±4.1% *versus* 6.2±4.4, $p=0.34$) following transradial catheterisation.

In summary radial FMD is impaired early after transradial catheterisation but appears to recover by 3 months. Whilst test-retest variability was demonstrated, our findings confirm the feasibility of using the radial artery following transradial cardiac catheterisation as model of vascular injury and repair *in vivo* in man.

3.2 INTRODUCTION

Difficulty in translating promising findings from pre-clinical models into the clinical arena (Hackam *et al.* 2006) is arguably exacerbated by a lack of human *in vivo* models to guide translational research and improve our understanding of human pathophysiology. An *in vivo* model to examine vascular injury and repair would afford mechanistic insights as well as the opportunity to test novel therapeutic approaches. While there are numerous animal models of both atherosclerotic and mechanical injury (Li *et al.* 2008; Tanaka *et al.* 2008; Silvestre *et al.* 2000; Jacobi *et al.* 2004; Moore *et al.* 1982; Strauss *et al.* 1994; Rodriguez-Menocal *et al.* 2008), inter-species differences and imperfect replication of prevailing clinical conditions limit their direct translation to humans.

Although rarely resulting in clinically relevant sequelae, transradial catheterisation is associated with subclinical abnormalities of radial artery structure (Yonetsu *et al.* 2010; Di Vito *et al.* 2013) and function (Yan *et al.* 2010; Dawson *et al.* 2010; 2012; Burstein *et al.* 2007) as a consequence of the trauma of intraluminal sheath insertion. The routine use of transradial access in clinical care and the accessibility of the human radial artery to non-invasive imaging provide a unique opportunity to study the mechanisms of vascular injury and repair *in vivo* in humans.

Flow-mediated dilatation of the brachial artery is widely established as a tool to assess vasomotor function *in vivo* in man (Corretti *et al.* 2001). Whilst brachial artery FMD is well characterised in terms of reproducibility, radial FMD is not. This may limit its utility in this setting of a potential clinical experimental medicine model of arterial injury. The aim of this study was to determine the reproducibility of FMD and nitrate-mediated dilatation in the assessment of radial artery function, and to examine the temporal effect of transradial catheterisation on radial artery vasomotor function.

3.3 METHODS

3.3.1 Subjects

Twenty healthy volunteers and 30 patients undergoing diagnostic transradial cardiac catheterisation for stable angina were recruited. The project was approved by the research ethics committee and written informed consent was obtained from all participants.

Subjects were assessed at the same time of day and asked to avoid food for four hours, and caffeine, vasoactive medications, smoking and alcohol for 24 hours prior study visits. All assessments were carried out in a quiet, temperature-controlled room and patients rested for 10 minutes before the first study measurements were made.

3.3.2 Study Protocols

3.3.2.1 Protocol 1

Twenty healthy volunteers and 20 patients attended on two occasions at least 24 hours apart. FMD and NMD of the left radial artery were assessed on two occasions per visit with at least 1 hour between repeat assessments.

3.3.2.2 Protocol 2

Ten patients undergoing elective cardiac catheterisation via the radial artery attended on 3 occasions (baseline and 24 h and 3 months following cardiac catheterisation). FMD and NMD of the radial artery were assessed in the catheterised (right) arm at each visit.

3.3.3 Flow- and Nitrate-mediated Dilatation

FMD and NMD were carried out as per international guidelines (Corretti *et al.* 2001; Thijssen *et al.* 2011). Briefly, the radial artery was imaged 5 cm proximal to the radial styloid with a 12 MHz linear-array ultrasound transducer (CX50 Phillips Amsterdam, Netherlands) held in place by a stereotactic clamp. A baseline recording was captured over 60 s. A supra-systolic cuff was then inflated to 220 mmHg for 5 minutes immediately distal to the antecubital fossa. Following release of the cuff, the artery was imaged continuously for five minutes (FMD). After 15 minutes of rest, the artery was once again imaged at rest for 60 s. Subjects were then given 25 µg of sublingual nitrate and the radial artery imaged for a further 5 minutes (NMD). This process was repeated after a one-hour rest period during which the subject was disconnected from the equipment and mobilised.

3.3.4 Image Acquisition (B-mode ultrasound)

Image acquisition was ECG-gated and arterial diameter captured during end-diastole (R-wave triggered). The artery was initially identified using colour-flow mapping. The probe position which gave the largest arterial diameter and clearest definition of the anterior vessel wall was chosen to minimise under-estimation of lumen diameter. The focus position of the probe was set to the anterior vessel wall.

3.3.5 Image Analysis (B-mode ultrasound)

Images were analysed offline using proprietary software (Brachial Analyser, Vascular Tools, Medical Imaging Applications, Iowa City, Iowa). Baseline and peak diameters were measured in millimetres with measurements averaged across sixty frames and ten frames for baseline and peak values respectively. Flow-mediated dilatation and NMD were expressed as percentage change in diameter from baseline.

3.3.6 Statistical Analysis

Results are reported as mean and standard deviation (SD) unless otherwise stated, reproducibility data is reported as mean bias with 95% limits of agreement. Comparisons between groups were made using Student's *t*-test.

Bland-Altman plots were used to examine reproducibility using data obtained from the left radial artery. Comparisons between time-points in protocol 2 were made using one-way repeated measures ANOVA. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA).

3.4 RESULTS

3.4.1 Subjects

All subjects (Table 1) tolerated the procedures well. There was no change in mean heart rate or systolic blood pressure following flow or nitrate-mediated dilatation.

| | Protocol 1 | | Protocol 2 |
|--------------------------------------|------------|------------|------------|
| | Healthy | | |
| | Patients | volunteers | Patients |
| | n=20 | n=20 | n=10 |
| Age | 61± 9 | 22±4 | 69±9 |
| Female (%) | 15 (75) | 13 (65) | 3 (30) |
| Heart Rate | 62±10 | 70±12 | 64±9 |
| Systolic Blood Pressure | 138±12 | 118±9 | 135±17 |
| Smoker (%) | 4 (20) | 0 (0) | 3 (30) |
| Coronary artery disease (%) | 18 (90) | 0 (0) | 7 (70) |
| Diabetes (%) | 4 (20) | 0 (0) | 2 (20) |
| Hypertension (%) | 15 (75) | 0 (0) | 90 (90) |
| Hyperlipidaemia | 15 (75) | 0 (0) | 70 (70) |
| Statin | 20 (100) | 0 (0)- | 9 (90) |
| Aspirin | 19 (95) | 0 (0)- | 10 (100) |
| Beta-blocker | 12 (60) | 0 (0)- | 6 (60) |
| Nitrate | 6 (30) | 0 (0)- | 4 (40) |
| Baseline radial artery diameter (mm) | 2.7±0.4 | 2.7±0.4 | 2.8±0.5 |

n(%), mean ±standard deviation

Table 1. Baseline characteristics of study populations

3.4.2 Flow- and Nitrate-mediated Dilatation

3.4.2.1 Arterial diameter

Baseline radial artery diameter was 2.7 ± 0.4 and 2.7 ± 0.4 mm in the 20 patients and 20 healthy volunteers respectively. In cohort 2 there was no change in arterial diameter following catheterisation with measurements of 2.8 ± 0.4 , 2.83 ± 0.37 and 2.82 ± 0.39 mm at baseline, 24h and 3 months respectively. Repeated measurements of baseline radial artery diameter demonstrated good reproducibility with intra-day coefficient of repeatability (CR) of 0.35 and 0.45 and inter-day CR of 0.61 and 0.73 in patients and healthy volunteers respectively.

3.4.2.2 Dilatation expressed as a percentage of baseline

There were no differences in percentage FMD (13.4 ± 6.4 *versus* $12.89 \pm 5.5\%$) or NMD (13.7 ± 3.8 *versus* $10.2 \pm 4.4\%$) between healthy volunteers and patients respectively (Figure 1). There were no differences in the magnitude of response for repeat testing within or between days in either FMD or NMD ($p > 0.05$ for all comparisons).

In healthy volunteers, the mean of the differences for within and between day measures (bias) was 1.99% (95% confidence intervals (CI), -12.5 to 16.5%) and 3.2% (95% CI, -13.8 to 7.5%) respectively for FMD and 1.8% (95% CI, -12.0 to 15.7%) and 1.7% (95% CI, -8.7 to 12.1%) respectively for NMD. In patients, the mean of the differences for within and between day measures was 2.53% (95% CI, -15.5 to 20.5%) and -4.3% (95% CI, -18.3 to 9.7%) respectively for FMD and 0.7% (95% CI, -12.1 to 13.6) and 1.7% (95% CI, -13.9 to 17.3%) respectively for NMD (Figures 2 and 3).

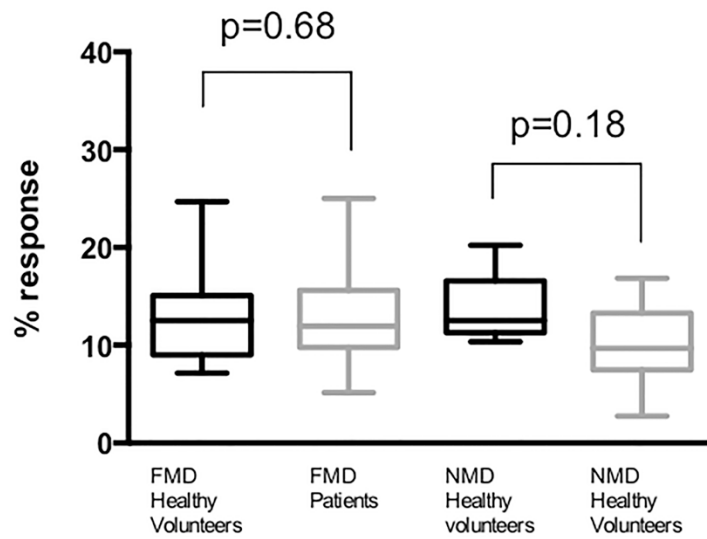


Figure 1

Baseline flow and nitrate mediated dilatation in patients and healthy controls.

Baseline flow-mediated dilatation (FMD) response in healthy volunteers shows no difference from that seen in patients (13.4 ± 6.4 versus $12.9 \pm 5.5\%$, $p=0.68$). Baseline nitrate-mediated dilatation (NMD) response was also similar in healthy volunteers and patients ($13.7 \pm 3.8\%$ versus 10.2 ± 4.4 respectively, $p=0.18$).

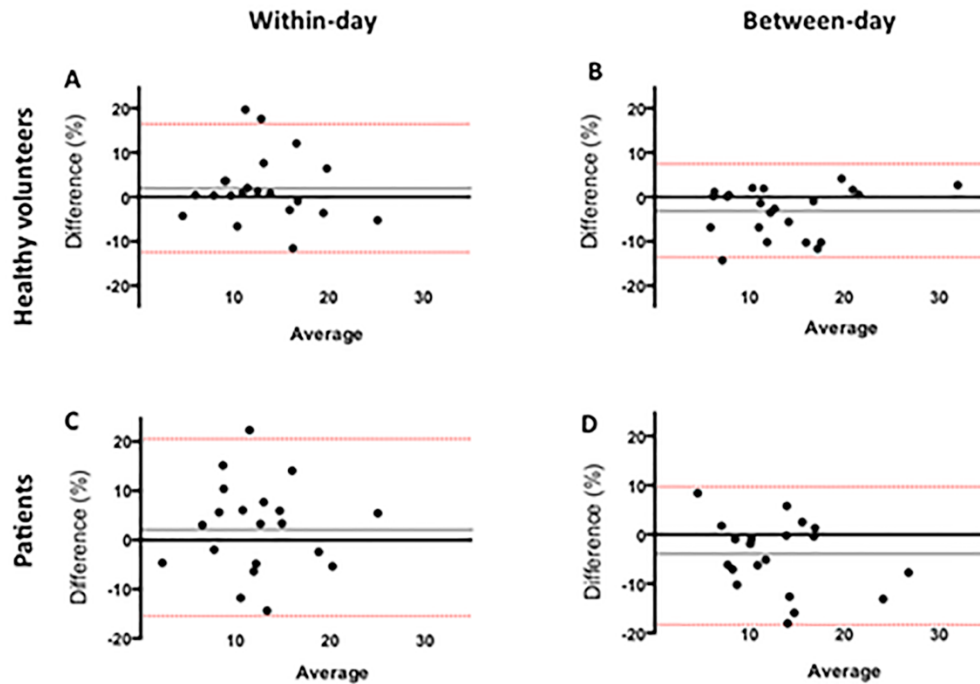


Figure 2

Reproducibility in percentage flow-mediated dilatation.

Within (A and C) and between (B and D) day variability in healthy volunteers patients. The continuous grey line represents the mean of the differences of the two measurements (the mean bias) and the red lines represent the 95% confidence intervals (limits of agreement).

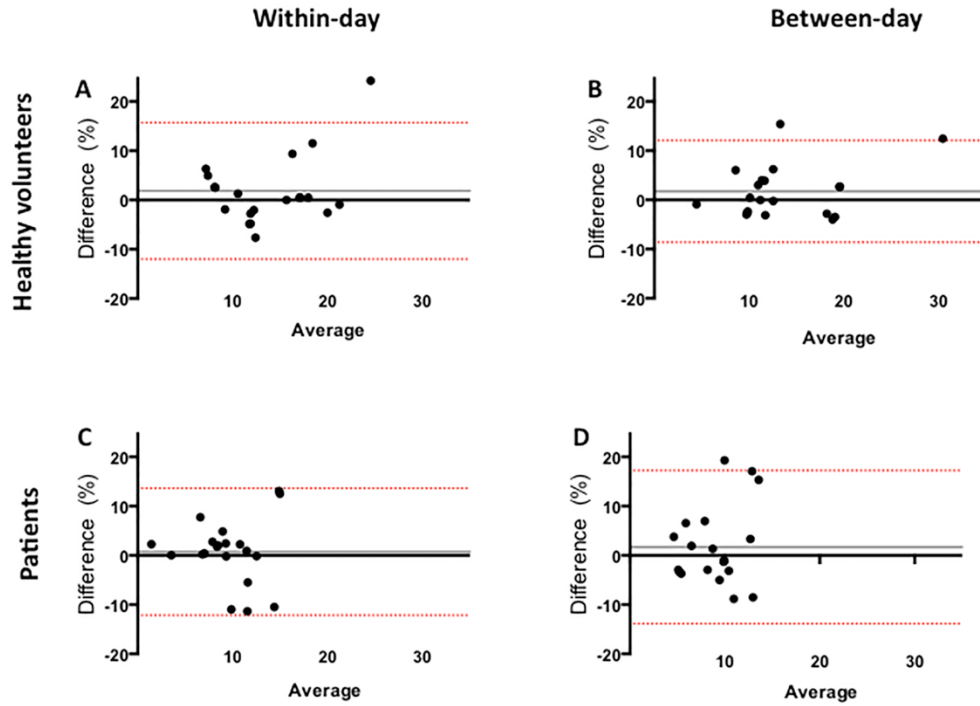


Figure 3

Reproducibility in percentage nitrate-mediated dilatation.

Within (A and C) and between (B and D) day variability in healthy volunteers patients. The continuous grey line represents the mean of the differences of the two measurements (the mean bias) and the red lines represent the 95% confidence intervals (limits of agreement).

3.4.2.3 Dilatation as absolute change in vessel diameter (mm)

There were no differences in absolute FMD (0.3 ± 0.3 versus 0.2 ± 0.2 mm) or NMD (0.3 ± 0.3 versus 0.2 ± 0.2 mm) between healthy volunteers and patients respectively. For both FMD and NMD there were no differences in the magnitude of responses for repeat testing within or between days ($p > 0.05$ for all comparisons).

In healthy volunteers, the mean of the differences for within and between day measures (bias) was -0.01 mm (95% CI, -0.32 to 0.38 mm) and 0.03 mm (95% CI, -0.3 to 0.31 mm) respectively for FMD and 0.02 mm (95% CI, -0.30 to 0.33 mm) and 0.03 mm (95% CI, 0.35 to 0.38 mm) respectively for NMD. In patients, the mean of the differences for within and between day measures was 0.01 mm (95% CI, -0.3 to 0.35 mm) and -0.02 mm (-0.35 to 0.38 mm) respectively for FMD and 0.03 mm (95% CI, -0.35 to 0.40 mm) and 0.02 mm (95% CI, -0.30 - 0.35 mm) respectively for NMD (Figures 4 and 5).

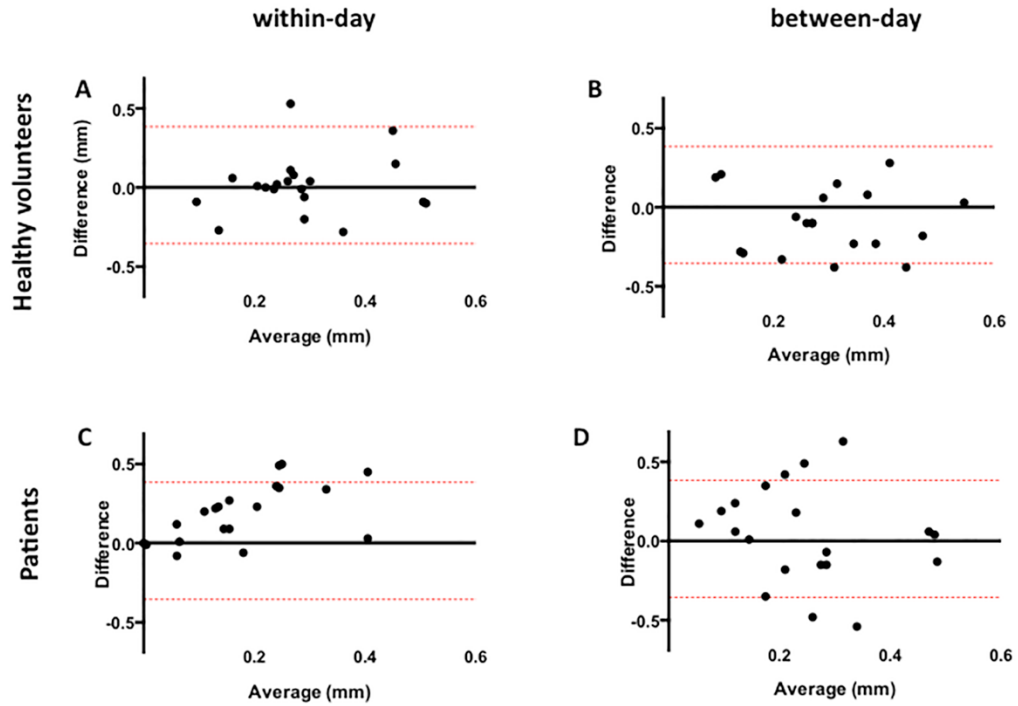


Figure 4

Reproducibility in absolute flow-mediated dilatation.

Within (A and C) and between (B and D) day variability in healthy volunteers patients. The continuous grey line represents the mean of the differences of the two measurements (the mean bias) and the red lines represent the 95% confidence intervals (limits of agreement).

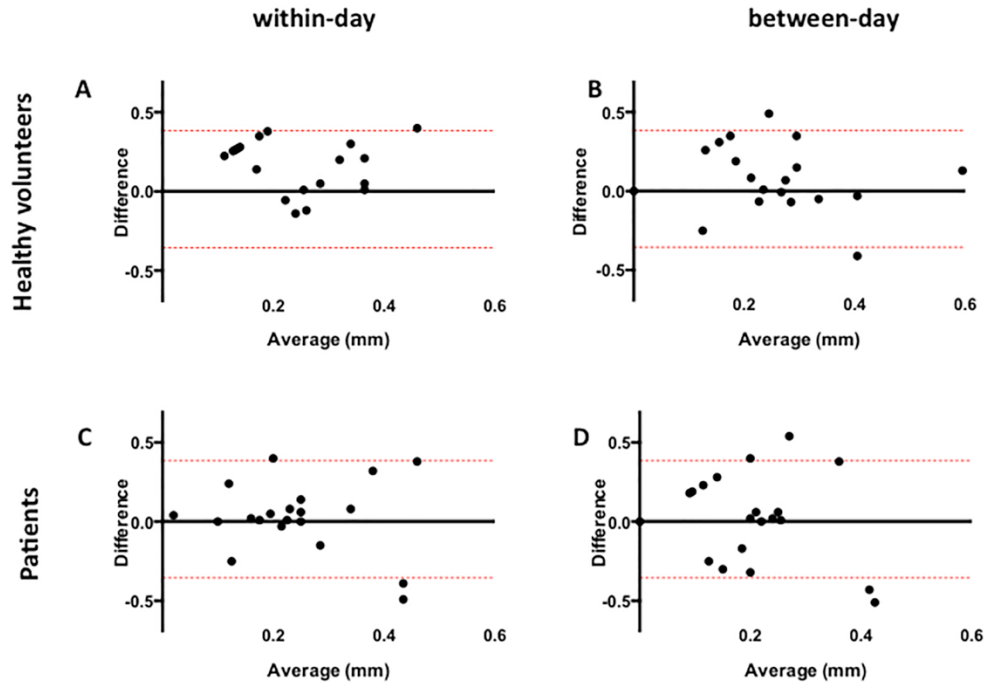


Figure 5

Variability in absolute nitrate-mediated dilatation.

Within (A and C) and between (B and D) day variability in healthy volunteers patients. The continuous grey line represents the mean of the differences of the two measurements (the mean bias) and the red lines represent the 95% confidence intervals (limits of agreement).

3.4.3 Arterial function following catheterisation (cohort 2)

In the 10 patients undergoing cardiac catheterisation, baseline radial diameter was 2.8 ± 0.5 mm (Table 1). Compared to baseline, radial artery FMD was impaired at 24 h (8.6 ± 4.0 % *versus* 3.9 ± 2.9 %, $p=0.015$) but not at 3 months (8.6 ± 4.0 % *versus* 6.2 ± 4.4 %, $p=0.34$; Figure 6) following transradial catheterisation.

As with FMD, compared to baseline NMD was impaired at 24 h (8.7 ± 5.5 % *versus* 2.8 ± 2.5 %, $p=0.006$). Response to nitrates remained impaired at 3 months (8.7 ± 5.5 % *versus* 4.8 ± 2.7 %, $p=0.047$; Figure 6) following transradial catheterisation.

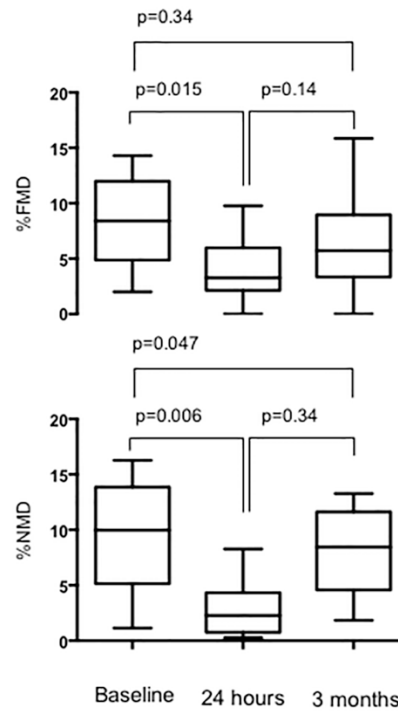


Figure 6

Flow and nitrate mediated dilatation in following radial artery catheterisation.

Flow-mediated dilatation (FMD) in the catheterised right radial artery was impaired at 24 h ($3.9 \pm 2.9\%$) when compared to baseline ($8.6 \pm 4.0\%$, $p=0.015$). There was some degree of functional recovery by three months ($6.2 \pm 4.4\%$) by which time FMD was not significantly different to baseline ($p=0.34$). Nitrate-mediated dilatation (NMD) was similarly impaired at 24 h ($2.8 \pm 2.5\%$) when compared to baseline ($9.4 \pm 5.1\%$, $p=0.006$). Again there was some improvement by 3 months ($4.8 \pm 2.7\%$) but this had not returned to baseline ($p=0.047$).

3.5 DISCUSSION

We have here demonstrated that FMD and NMD can be used to assess radial artery vasomotor function in man with acceptable reproducibility. Using this technique, we have demonstrated that radial artery vasomotor function is impaired early after transradial access for cardiac catheterisation, but appears to recover, at least in part, by 3 months with endothelium-dependent vasodilatation no longer significantly different from baseline. Whilst impairment of radial function post-catheterisation has previously been described, reproducibility of this technique is not well characterised. Adding to the work of Brook et al (Brook *et al.* 2005) who characterised reproducibility in healthy volunteers, we have also made an assessment in a patient cohort. Our findings suggest that transradial access for cardiac catheterisation may afford a potential model of vascular injury and repair *in vivo* in man.

Previous studies have demonstrated that the heterogeneity observed in the FMD response is dependent in part on the flow stimulus (Mullen *et al.* 2001; Agewall *et al.* 2001) as well as underlying physical characteristics of subjects (Mullen et al. 2001). To address these potential concerns, we standardised the environmental conditions and experimental protocol although we did not document the flow stimulus by Doppler ultrasound. We observed minimal within and between day variability in the measurement of absolute radial artery diameter using ultrasound. However, we did observe more prominent

heterogeneity in the relative responses to FMD and NMD in both healthy volunteers and patients.

The variability in radial FMD is more pronounced than the more widely used brachial FMD for which a coefficient of variation of 1.8% has previously been reported in the original study by Sorensen *et al* (Sorensen *et al.* 1995). However, the calculation of this variability was unclear and whilst subsequent studies have reported that baseline brachial artery diameter can be measured reproducibly, most report a coefficient of variation for brachial FMD of between 10% and 50% (Liang *et al.* 1998; De Roos *et al.* 2001).

Although the resting diameter of the radial artery is smaller than the brachial artery, vasodilatation causes similar absolute increases in arterial diameter with both arteries. This has some important effects on the calculation of the relative diameter changes for FMD and NMD. In other words, the percentage change in vessel size is larger for radial than brachial FMD because of the smaller denominator. Whilst superficially this is attractive for the detection of changes due to therapeutic interventions, inaccuracies will be exaggerated and reproducibility compromised. The lack of linearity between baseline diameter and absolute FMD response has been suggested as a confounder in comparisons between and within subjects. Atkinson *et al* (Atkinson *et al.* 2013). suggest a promising way of correcting for this although this is yet to be widely adopted. Ultimately our data demonstrate that both absolute and relative changes in

radial FMD and NMD are more variable than those seen for the brachial artery and this is consistent with previous work (Brook *et al.* 2005).

Dilatation of the radial artery in the human forearm in response to short periods of ischaemia is dependent on an intact endothelium and is attenuated by infusion of the nitric oxide synthase inhibitor, *N*^Gmonomethyl-L-arginine (Mullen *et al.* 2001). Acute disruption of the vessel wall has been demonstrated following transradial cardiac catheterisation using optical coherence tomography-based intravascular imaging (Yonetsu *et al.* 2010; Di Vito *et al.* 2013). The catheterised radial artery has also been examined histologically at the time of conduit harvest for coronary artery bypass grafting with one study demonstrating endothelial disruption, with the degree of endothelial loss inversely related to the time since catheterisation (Kamiya *et al.* 2003), although this has not been a universal finding (Gaudino *et al.* 2015). It is perhaps not surprising therefore, that we and others (Dawson *et al.* 2010; Madssen *et al.* 2006; Okuyan *et al.* 2012; Park *et al.* 2012) have demonstrated that transradial catheterisation results in impairment of FMD of the radial artery. This likely represents endothelial denudation with recovery of vasomotor function indicating reconstitution of the monolayer. The attenuation of both FMD and NMD after radial catheterisation is in keeping with previous studies (Dawson *et al.* 2010; Burstein *et al.* 2007) and implies that the vascular injury involves both the vascular smooth muscle layer and the superficial endothelial layer. The time course of recovery remains unclear with some authors reporting complete recovery of radial artery vasomotor function (Yan *et al.* 2010; Dawson *et al.*

2010; Tilling *et al.* 2014) whilst others observed irreversible impairment (Burstein *et al.* 2007). Our data would suggest that there is some recovery of function at three months but we cannot be confident of complete restoration of function.

Previous studies have used the radial artery in the context of cardiac catheterisation to examine the influence of factors such as sheath coating (Dawson *et al.* 2010) and drug therapy (Li *et al.* 2008; Park *et al.* 2012; Turan *et al.* 2015) on endothelial recovery. These models have however only examined forearm vasomotion and at a limited number of time points. Understanding the exact mechanism, the time course of injury and its recovery is critical if this model is to realise its potential in the study of vascular injury and repair *in vivo* in man.

3.6 CONCLUSION

Whilst radial FMD is a technique with inherent variability, the larger magnitude of the baseline response compared to brachial FMD and the profound impact of catheterisation on endothelial function mean that the effect of arterial injury can be demonstrated in a small cohort of patients. This, combined with the ubiquitous use of radial access and the non-invasive nature of FMD, implies transradial cardiac catheterisation may be a powerful and accessible tool for studying mechanical vascular injury. This preliminary study suggested the feasibility of the radial artery as a model which may permit mechanistic insights

into the processes of vascular injury and repair as well as the modifying influences of cardiovascular risk factors and therapies.

Chapter 4

Radial artery injury and recovery following transradial cardiac catheterisation

Data from this chapter is published in: *The Journal of the American Heart Association*:

Mitchell AJ, Fujisawa T, Mills NL, Brittan M, Newby DE, Cruden NL 'Endothelial progenitor cell biology and vascular recovery following transradial cardiac catheterisation' *JAHA* (2017);**6**:e006610.

4.1 SUMMARY

Transradial catheterisation causes subclinical radial artery injury and impaired vascular function. The mechanism, extent and resolution of this injury remain unclear. This study aimed to comprehensively characterise the frequency and severity of radial artery injury and the time course of local vasomotor dysfunction and recovery, thus allowing exploration of the cellular responses to vascular injury and their influences on vessel recovery (**Chapter 5**).

Patients undergoing elective transradial cardiac catheterisation were studied (n=50). Radial artery injury was characterised using optical coherence tomography and radial arterial sheaths were examined for the presence of endothelial cells. Flow- and nitrate-mediated dilatation of the radial artery were assessed bilaterally at baseline, 24 h and 1, 4 and 12 weeks. Blood samples were collected for circulating progenitor cell enumeration and Late outgrowth endothelial cell isolation (**Chapter 5**).

Macroscopic injury was identified in 12 patients (24%). Endothelial cells (mean $1.9 \pm 1.2 \times 10^4$ cells/sheath) were isolated from all arterial sheaths examined. Compared to the non-catheterised radial artery, flow-mediated ($9.9 \pm 4.6\%$ *versus* $4.1 \pm 3.1\%$, $p < 0.0001$) and nitrate-mediated ($8.8 \pm 5.4\%$, *versus* $2.9 \pm 4.7\%$, $p < 0.0001$) vasodilatation were impaired in the catheterised artery at 24 h.

Nitrate-mediated vasodilatation was similar in the catheterised and non-catheterised arteries by 4 weeks ($6.6 \pm 4.1\%$ *versus* $8.4 \pm 3.4\%$, $p=0.12$) and flow-mediated vasodilatation by 12 weeks ($8.1 \pm 4.9\%$ *versus* $10.1 \pm 4.9\%$, $p=0.09$).

Transradial cardiac catheterisation causes endothelial injury and vasomotor dysfunction which recovers over time. This offers an easily accessible and reproducible model of *in vivo* arterial injury in man and the opportunity to gain mechanistic insights into the processes of vascular injury and repair and examine the modifying effects of novel therapies.

4.2 INTRODUCTION

Endothelial injury is a critical initiating event in atherosclerosis and ubiquitous following high-pressure balloon inflation during percutaneous coronary intervention (Grewe *et al.* 2000). The consequence of this injury is to a large degree determined by the regenerative responses of the vasculature. Efforts to understand and augment this response *in vivo* have been hampered by the lack of a comprehensively characterised model of mechanical injury in man.

Whilst ethical considerations preclude the induction significant experimental arterial injury in man, transradial cardiac catheterisation offers the opportunity to study this process in a stable outpatient population. The radial artery has emerged as the access site of choice for coronary angiography ('BCIS National Audit of Percutaneous Coronary Interventions' 2014) with greater patient

comfort (Cooper *et al.* 1999), reduced length of stay and improved outcomes (Cruden *et al.* 2007; Mamas *et al.* 2012). Structural injury to the vessel is however commonplace (Yonetsu *et al.* 2010; Di Vito *et al.* 2013) as is functional impairment as assessed by flow-mediated dilatation (Madssen *et al.* 2006; Dawson *et al.* 2010; Gaudino *et al.* 2015; Okuyan *et al.* 2012; Park *et al.* 2012; Burstein *et al.* 2007). Whilst seldom associated with clinical sequelae, these changes afford an opportunity to study vascular injury *in vivo* in man.

.

This study aimed to characterise in detail the extent of acute structural injury following transradial catheterisation, the resultant degree of functional impairment and the time course of vessel recovery.

4.3 METHODS

4.3.1 Subjects

Fifty patients undergoing elective transradial cardiac catheterisation for known or suspected coronary artery disease at the Royal Infirmary of Edinburgh were recruited. Patients without bilaterally palpable radial arteries, those experiencing a recent acute coronary syndrome or recent cardiac catheterisation (<3 months), severe valvular heart disease, renal impairment (estimated glomerular filtration rate <30ml/min), contrast allergy, pregnancy, inability to give informed consent and those aged below 18 or above 85 years

were excluded. The study was approved by the local research ethics committee and written informed consent was obtained from all patients.

4.3.2 Study visits

The study visits are outlined in **figure 1**. Structural injury to the artery was assessed by optical coherence tomography (OCT) at the time of angiography. Radial sheaths were also examined for endothelial cells in a subset of patients (n=10). Function of the radial artery was assessed using flow- and nitrate-mediated dilatation (FMD and NMD, respectively) at baseline, 24 hours, 1, 4 and 12 weeks post-angiography. In all cases the uninjured left radial artery was examined as an internal control.

| | | | |
|----------|------------------------------|-------------------|--|
| Baseline | Flow-mediated dilatation | Venesection* | 50 patients |
| | Optical coherence tomography | Angiogram +/- PCI | 50 patients |
| 24 hours | Flow-mediated dilatation | Venesection* | 48 patients (2 radial artery occlusions) |
| 1 week | Flow-mediated dilatation | | 48 patients |
| 4 weeks | Flow-mediated dilatation | | 47 patients (1 repeat catheterisation) |
| 12 weeks | Flow-mediated dilatation | | 45 patients (2 early coronary artery bypass) |

* 100 mL Blood sample for analysis of circulating endothelial progenitor cell concentrations and endothelial outgrowth cell isolation.

Figure 1: Study Protocol. Flow-mediated dilatation and nitrate-mediated dilatation was assessed bilaterally at each visit and peripheral blood samples were taken at baseline and 24 h post-angiography. Numbers of patients completing follow-up at each stage is outlined in the right-hand column with reasons for attrition in parentheses.

PCI: percutaneous coronary intervention.

4.3.3 Coronary angiography

Angiography was performed via the right radial artery in all cases. Briefly, following local anaesthesia with 1% lidocaine the radial artery was punctured using Seldinger technique. Heparin was administered when access to the aortic root was confirmed: 5000 units bolus with additional boluses at operator's discretion if follow-on percutaneous coronary intervention was undertaken. Arterial sheaths were removed at the end of the procedure with haemostasis achieved using a TR band™ (Terumo, Japan).

4.3.4 Optical coherence tomography

4.3.4.1 Image acquisition (optical coherence tomography)

Optical Coherence Tomography of the radial artery was performed at the time of diagnostic angiography. Briefly, a 6-French arterial sheath (Check-Flo™ 13cm, Cook medical, USA) was inserted a short distance (2 cm) into the vessel. A coronary guide wire (Balance Middleweight, Abbot Vascular, USA) was then passed to the brachial artery under fluoroscopic guidance over which an OCT catheter (Fastview™ Terumo, Japan) was advanced with position monitored using live intravascular imaging. Following 200µg of intra-arterial glyceryl trinitrate, contrast (75% solution Omnipaque™, GE healthcare, USA) was then injected into the radial sheath and pullback of the 15 cm of artery proximal to sheath insertion performed. The sheath was then inserted to its full length and

the angiogram performed as usual. At the end of the case the sheath was withdrawn to its original 2 cm point and the artery imaged again as above.

4.3.4.2 Image analysis (optical coherence tomography)

Vessel dimensions were assessed at 1-cm intervals along the imaging run with three consecutive frames analysed at each location. Areas of the internal elastic lamina (IEL), external elastic lamina (EEL) and radial artery lumen were manually defined then automatically calculated using proprietary software (Lunawave, Terumo, Japan). Mean intimal area was defined as average internal elastic lamina area minus average luminal area. Structural injury was assessed qualitatively on a frame-by-frame basis and classified as, an intimal tear (a visible flap contained within the intima) or medial dissection (extending into the media) when present on at least three consecutive frames.

4.3.4.3 Measurements (optical coherence tomography)

Vessel dimensions were assessed at 1-cm intervals along the imaging run with three consecutive frames analysed at each location. Areas of the internal elastic lamina, external elastic lamina and radial artery lumen were measured using proprietary software (Lunawave, Terumo, Japan) and manual tracing. Mean intimal area (average internal elastic lamina area minus average luminal area) was calculated along the length of the vessel. Structural injury was assessed qualitatively with each imaging run analysed on a frame-by-frame basis.

Identified injuries were classified as, intimal tear (if a flap was visible but did not extend beyond the intima), or as medial dissection (if the injury extended into the smooth muscle layer). Only lesions present on at least three consecutive frames were adjudicated as injuries.

4.3.5 Cell recovery from arterial sheaths

In a subset of patients (n=10) arterial sheaths were examined for endothelial cells at the end of the procedure. The lumen of the sheath was flushed to remove blood and the sheaths were placed in phosphate-buffered saline and centrifuged at 5000 rpm for 10 minutes. Cells obtained were stained using the endothelial cell antigen CD31-PE (BD Biosciences, USA), platelet marker CD42a-FITC (BD Biosciences, USA) and haematopoietic cell antigen CD45-APC (BD Biosciences, USA) and 40,000 events were acquired using a 4-laser flow cytometer (LSR-Fortessa II, BD, USA). Gating strategy is outlined in **figure 2**.

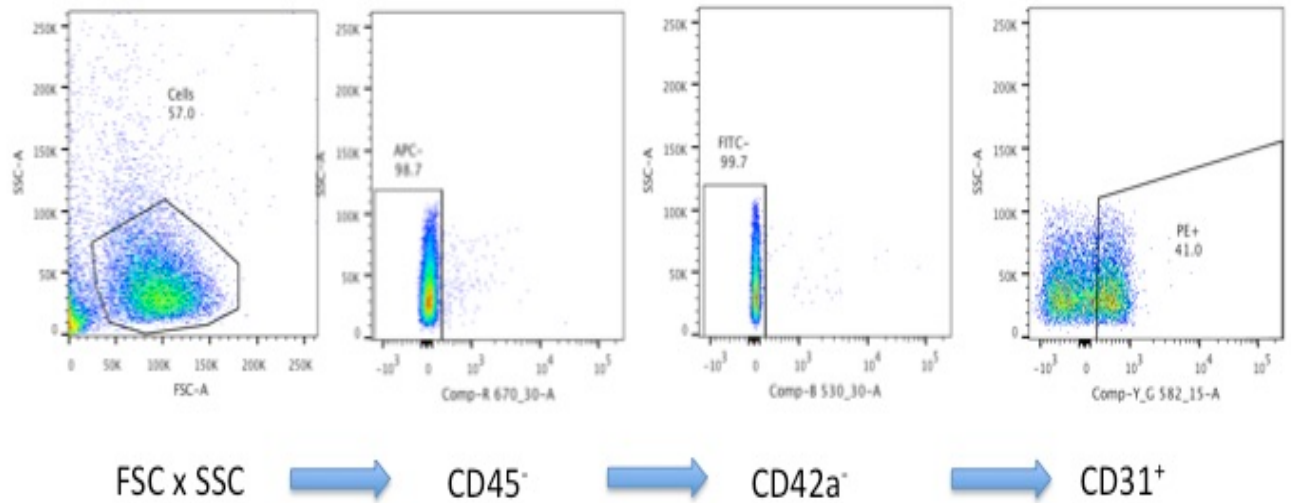


Figure 2: Gating strategy for endothelial cell enumeration from radial sheaths. Cells were excluded from debris using a forward/ side-scatter plot. Haematopoietic cells and platelets were then excluded by selecting CD45 and CD42a negative populations respectively. Endothelial cells were then identified as those positive for CD

4.3.5 Flow and nitrate-mediated dilatation

Flow and nitrate-mediated dilatation (FMD and NMD respectively) were carried out according to international guidelines (Corretti *et al.* 2001; Thijssen *et al.* 2011). Briefly, the radial artery was imaged 5 cm proximal to the radial styloid with a 12-15 MHz linear-array ultrasound transducer (CX50 Phillips, Netherlands) held in place by a stereotactic clamp. A baseline recording was captured over 60 seconds. A supra-systolic cuff was then inflated to 220 mmHg for 5 minutes immediately distal to the antecubital fossa. Following release of the cuff, the artery was imaged continuously for five minutes (FMD). After 15 minutes of rest, the artery was again imaged at rest for 60 seconds. Subjects were then given 25 µg of sublingual nitrate and the radial artery imaged for a further 5 minutes (NMD).

Subjects were assessed at baseline, twenty-four hours, one week, one month and three months. Participants were asked to avoid food for 4 h prior to study visits and caffeine, smoking and vaso-active medications for 24 h. Assessments were carried out in a quiet, temperature-controlled room at the same time of day to minimise biological variation.

4.3.5.1 Image acquisition (B-mode ultrasound)

Image acquisition was ECG-gated with arterial diameter captured during end-diastole (R-wave triggered). The artery was initially identified using colour-

flow mapping. The probe position that gave the largest arterial diameter and clearest definition of the anterior vessel wall was chosen to minimise under-estimation of the lumen. The focus position of the probe was set to the anterior vessel wall as this is the most challenging to resolve (Corretti *et al.* 2001).

4.3.5.2 Image analysis (B-mode ultrasound)

Images were analysed offline using proprietary software (Brachial Analyser, Vascular Tools, Medical Imaging Applications, USA). Baseline and peak diameters were measured in millimetres with measurements averaged across sixty frames and ten frames for baseline and peak values, respectively. Flow-mediated dilatation and NMD were expressed as percentage change in diameter from baseline.

4.3.6 Systemic haemodynamics

Mean arterial pressure and heart rate were monitored during the resting period and after cuff release.

4.3.7 Statistical analysis

Results are expressed as mean with standard deviation or median with inter-quartile range as appropriate. Comparisons between normally distributed

variables were made using paired and unpaired t-tests and ANOVA. Analyses were performed using SPSS version 21.0 (IBM Corporation, USA).

4.4 RESULTS

Fifty patients were enrolled in the study (**Table 1**). Subjects were predominantly male (70%) with a mean age of 64 ± 10 years. Eighteen patients (36%) underwent follow-on percutaneous intervention. Radial artery OCT images were available for diagnostic interpretation in forty-eight patients. Forty-five patients completed the radial artery protocol: one patient was excluded due to early repeat transradial cardiac catheterisation; two patients were excluded due to persistent radial occlusion post-procedure and two patients underwent early coronary artery bypass grafting.

Table 1: Clinical characteristics of study population (n=50)

| | |
|---|----------|
| Age | 64±10 |
| Male | 35(70%) |
| Hypertension | 37 (74%) |
| Diabetes | 7 (14%) |
| Current smoker | 10 (20%) |
| Hypercholesterolemia | 28 (56%) |
| Family history | 38 (76%) |
| Previous radial access | 7 (14%) |
| PCI | 18 (36%) |
| Total cholesterol (mg/dL) | 176± 8.5 |
| Creatinine (mg/dL) | 0.9±0.2 |
| C-reactive protein (mg/L) | 3.7±4.4 |
| Medications | |
| Aspirin | 46 (92%) |
| Clopidogrel | 30 (60%) |
| Beta-blocker | 41 (82%) |
| ACE-I/ ARB | 18 (36%) |
| Nitrate | 10 (20%) |
| Statin | 48 (90%) |
| Baseline Radial artery diameter (mm) | |
| Ultrasound | |
| Right | 2.9±0.4 |
| Left | 2.9±0.5 |
| OCT | |
| Right | 3.0±0.4 |

PCI= percutaneous coronary intervention, ACE-I= angiotensin-converting enzyme inhibitor, ARB= Angiotensin receptor-blocker, OCT=optical coherence tomography

4.4.1 Acute injuries to the radial artery

The radial artery was imaged by OCT over an average length of 132 ± 12 mm.

The radial artery diameter measured 3.2 ± 0.4 mm with a cross-sectional area of 10.7 ± 2.5 mm² and intimal area of 0.6 ± 0.2 mm². Macroscopic injury arising from transradial catheterisation was observed in 12/50 (24%) patients. Endothelial disruption limited to the intima was seen in 8 (16%) patients, whilst significant dissection extending into the media was seen in 4 (8%) patients (**Figure 3**).

Macroscopic injury was observed in the distal vessel in 6 (50%) patients, mid-vessel in 1 (8%) patients and proximal vessel in 5 (42%) patients. Endothelial cells defined as CD31⁺, CD42a⁻ and CD45⁻ were isolated from all arterial sheaths ($1.9 \pm 1.2 \times 10^4$ cells/sheath) irrespective of the presence or absence of macroscopic injury. Patients were defined as having evidence of acute vascular injury if injury was observed on OCT or if they underwent follow on PCI during the procedure.

4.4.2 Flow- and nitrate-mediated dilatation

Radial artery diameter at baseline was 2.9 ± 0.4 mm and 2.9 ± 0.5 mm in the catheterised and non-catheterised arteries respectively. FMD was impaired in the catheterised radial artery at 24 h ($4.1 \pm 3.1\%$) compared to the non-catheterised artery ($9.9 \pm 4.6\%$, $p < 0.0001$). At 3 months FMD was no longer significantly impaired in the catheterised when compared to the non-

catheterised radial artery ($8.1 \pm 4.9\%$ *versus* $10.1 \pm 4.9\%$ respectively, $p=0.09$; Figure 4). NMD in the catheterised radial artery at 24 h was attenuated when compared to the non-catheterised radial artery ($2.9 \pm 4.7\%$ *versus* $8.8 \pm 5.4\%$ respectively, $p<0.0001$). This dysfunction persisted at one week but was no longer present at one month ($6.6 \pm 4.1\%$ *versus* $8.4 \pm 3.4\%$ for catheterised *versus* non-catheterised, respectively, $p=0.12$;) (**Figure 4**).

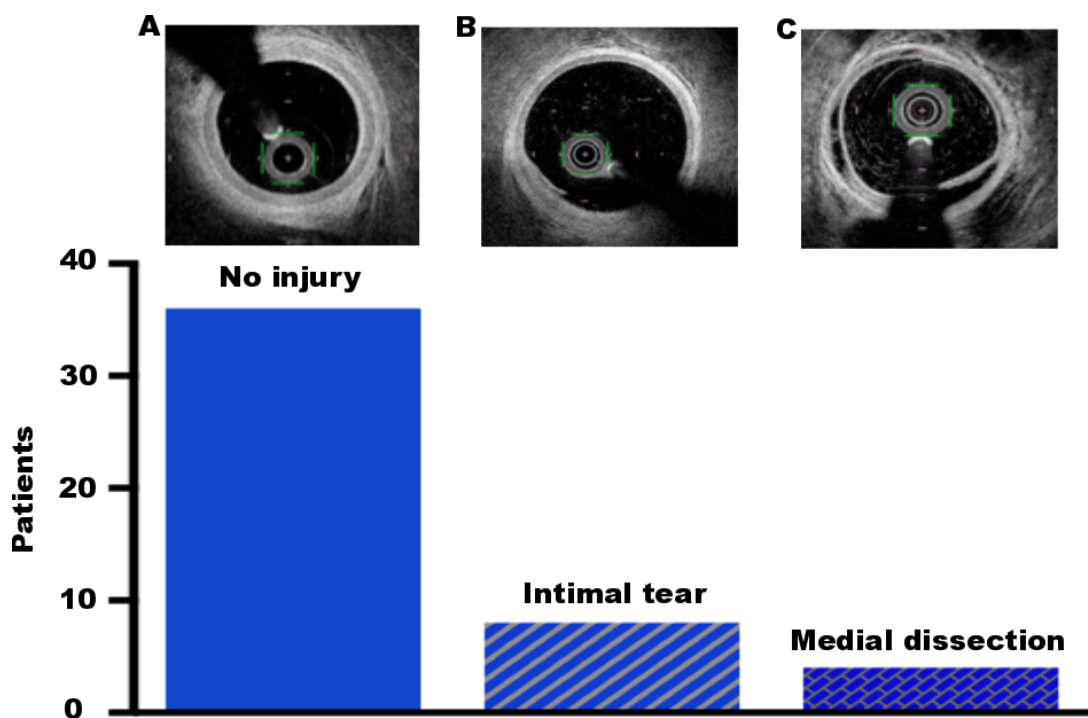


Figure 3: Structural injury to the radial artery following cardiac catheterisation. No acute injury was detectable on intravascular imaging in the majority of patients. Normal artery at sheath withdrawal **(A)**. Small intimal tear at seven o'clock position **(B)**. Extensive circumferential dissection extending into the media **(C)**.

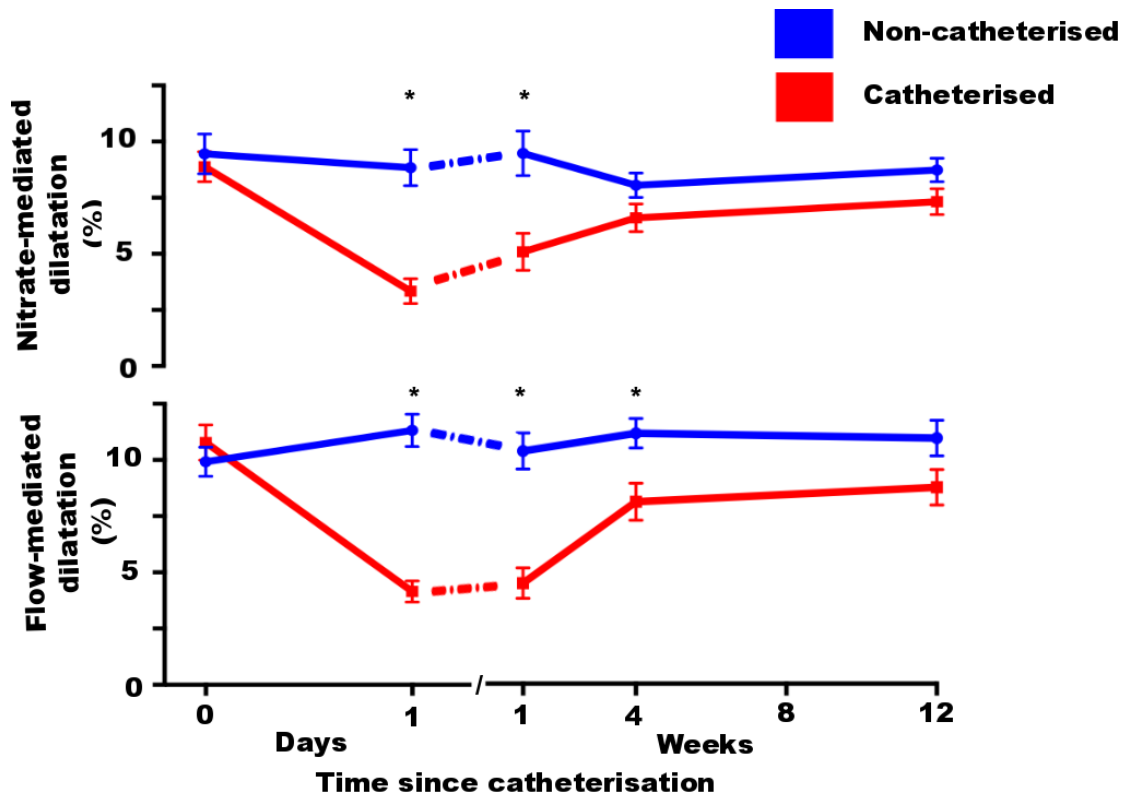


Figure 4: Changes in radial artery FMD and NMD post-catheterisation.

Flow-mediated dilatation in the catheterised radial artery was impaired at 24 h, one and four weeks when compared to the uninjured radial artery but recovered by three months. Nitrate-mediated dilatation was impaired at 24 h and one week but by four weeks there was no longer a significant difference between injured and uninjured radial arteries. Red lines represent the injured right radial artery with blue lines representing the (uninjured) left radial artery. Mean and 95% confidence intervals displayed.

*p<0.05 for right vs left comparison.

4.5 DISCUSSION

This study comprehensively characterised the structural and vasomotor changes in the radial artery which accompany transradial cardiac catheterisation. Whilst there was a surprisingly low incidence of macroscopic arterial injury, endothelial denudation was reliably demonstrated and this was associated with vasomotor impairment which recovered, although incompletely, over three months.

Our study confirms the safety of transradial catheterisation with a low incidence of macroscopic injury identified by OCT. Two previous studies have examined radial injury following cardiac catheterisation with Yonetsu *et al* (Yonetsu *et al.* 2010) demonstrating acute injuries in 67% of patients and Di Vito *et al* (Di Vito *et al.* 2013) reporting an incidence of 37%. In both cohorts, there were high numbers of patients attending for a repeat catheterisation which in the Yonetsu study was the only predictor of arterial injury on multivariable analysis. It is likely that a low incidence of repeat procedures and advances in sheath technology as well as racial difference in radial artery calibre in the Yonetsu study (which was conducted in a Japanese population) explain the divergent rates of acute vascular injury.

Whilst the incidence of macroscopic arterial injury was low, sheath removal was reliably associated with extensive denudation of the local endothelial cell layer.

Microscopic endothelial disruption post-catheterisation has been demonstrated previously by histological examination of radial arteries at the time of harvest for bypass grafting and appears to recover over time (Gaudino *et al.* 2015). This likely explains the functional impairment seen in our study with functional recovery reflecting re-endothelialisation. It is not known whether re-endothelialisation occurs as a result of local ingrowth or recruitment of circulating endothelial progenitor cells to the site of injury.

Previous work has documented radial artery function following cardiac catheterisation with the majority of studies reporting a recovery of function over a three-month period (Madssen *et al.* 2006; Park *et al.* 2012; Tilling *et al.* 2014; Dawson *et al.* 2010) although this has not been universal (Yan *et al.* 2010; Okuyan *et al.* 2012). The time course of NMD recovery following transradial catheterisation is less well documented in the literature although a reversible defect has been described in some studies (Dawson *et al.* 2010; Madssen *et al.* 2006). The more rapid recovery of NMD than FMD in our study suggests different pathophysiology between the layers of the arterial wall. The intima appears to suffer significant microscopic disruption with the media most likely suffering an acute inflammatory insult without significant structural disruption post catheterisation. Medial smooth muscle function recovers relatively rapidly whilst the intima requires reconstitution of the endothelial monolayer and thus functional recovery is delayed. In keeping with our observations, Staniloae (Staniloae 2009) demonstrated medial and adventitial inflammation in radial

arteries post-catheterisation without significant disruption of the medial architecture.

4.6 CONCLUSION

Transradial cardiac catheterisation causes a transient depression of endothelium-dependent and independent vasomotion which despite low rates of injury detectable by intra-vascular imaging, appears to be due to endothelial denudation. Documenting the structural and functional changes in the radial artery following catheterisation alongside characterisation of the accompanying peripheral cellular profiles (**Chapter 5**) allowed examination of the cellular influences on vessel recovery following mechanical injury.

Chapter 5

Circulating Cellular profiles in vascular injury and repair

Data from this chapter is published in *the Journal of the American Heart Association*:

Mitchell AJ, Fujisawa T, Mills NL, Brittan M, Newby DE, Cruden NL 'Endothelial progenitor cell biology and vascular recovery following transradial cardiac catheterisation' *JAHA* 2017 **6**:e006610.

5.1 SUMMARY

Endothelial progenitor cells and late-outgrowth endothelial cells are thought to be important vessel repair following injury, their exact definition and role in this process remain controversial. Transradial cardiac catheterisation causes endothelial denudation and consequently a reversible impairment of vasomotor function.

Patients undergoing transradial cardiac catheterisation (n=50) were studied and the structural and functional consequences of the procedure were examined out to 3 months (Chapter 4). Peripheral blood was collected for enumeration of endothelial progenitor cells and endothelial outgrowth cell isolation at baseline and 24h post-catheterisation. EPC and EOC number and EOC function were examined to determine their effect on vessel function at baseline and on recovery following injury.

Baseline endothelial function was negatively correlated with baseline CD34⁺ ($r=-0.4$, $p=0.004$) and CD133⁺ ($r=-0.33$, $p=0.02$) cell concentrations. Recovery of arterial function was negatively correlated with baseline CD34⁺ concentration ($r=-0.33$, $p=0.04$). Neither baseline arterial function nor recovery post-injury correlated with the number of late outgrowth endothelial cell colonies isolated. There was a positive correlation between the migratory capacity of EOC, and baseline endothelial function ($r=0.47$, $p=0.03$), but not recovery following injury ($r=0.02$, $p=0.94$).

Heterogeneous populations of circulating cells such as those defined by markers of cellular naïveté such as CD133 or CD34 do not seem to enhance vascular repair but late-outgrowth endothelial cell function correlates with radial vasomotor function at baseline, underlining the importance of this cell population to vascular health.

5.2 INTRODUCTION

The cellular response to vascular injury is an important modifier of atherogenesis as well as vascular remodelling and vessel outcome following percutaneous coronary intervention (Gabbasov *et al.* 2009; Pietersma *et al.* 1995; Inoue *et al.* 2007). Since first described by Asahara *et al.* in 1997 (Asahara *et al.* 1997), there has been intense interest in endothelial progenitor cells, thought to be capable of homing to and repopulating areas of injured endothelium following vessel injury.

Early studies (Werner *et al.* 2005; Schmidt-Lucke *et al.* 2005) described robust correlations between EPC numbers and various cardiovascular outcomes. A recent meta-analysis (Rigato *et al.* 2016) again found a negative correlation between certain progenitor cell populations and cardiovascular outcomes, including restenosis following PCI, albeit with significant heterogeneity between studies and overall a less compelling relationship than reported in

initial studies. Controversy persists over the phenotypic definition of these cells and their specific role in vascular repair.

Typically defined by the presence of cell-surface antigens CD34 and KDR (the extracellular domain of the vascular endothelial growth factor receptor-2) with or without CD133 as an additional marker of cellular naiveté, EPCs are rare in health, comprising significantly less than 0.01% of circulating mononuclear cells (Peichev *et al.* 2000), making their study and therapeutic application challenging.

In contrast, late outgrowth endothelial cells (EOCs) may be a more attractive candidate for studies of vascular biology and therapeutic trials. They can be isolated from peripheral blood mononuclear cells under angiogenic culture conditions (Ingram *et al.* 2004), have high proliferative activity and form a monolayer of cells resembling mature endothelium in culture (Ingram *et al.* 2004; Yoder 2009). EOCs are thought to represent the end product of EPC mobilisation and are more amenable to *in vitro* analysis than putative EPCs defined by cell surface phenotype alone.

Given their presumed central role in vessel repair we sought to define both the effect of vascular injury on EPC and EOC populations and the modifying effect if any that these populations have on vessel recovery.

5.3 METHODS

50 patients were studied at baseline and out to three months following elective outpatient coronary angiography as detailed in **(Chapter 4)**. Characterisation of the structural and functional changes in the radial artery following catheterisation are outlined in **(Chapter 4)**. Cellular populations were characterised at baseline and 24 hours post-catheterisation as described below.

5.3.1 Endothelial Progenitor Cell enumeration by flow cytometry

Venous blood (100 mL) was obtained from all patients at baseline and 24 hours post-angiography. Fresh blood was stained with panels of pre-conjugated anti-human monoclonal antibodies to quantify putative EPC and monocyte populations: EPC panel: CD45-V450 (BD horizon), CD34-APC/Cy7 (Biolegend, USA), KDR-PE (BD Biosciences, USA), CD133-APC (Miltenyl Biotec, Germany), KDR-PE(BD Biosciences, USA).

CountBright™ beads (ThermoFisher Scientific, UK) were used to quantify absolute cell numbers. Unstained samples were used as negative controls and compensation was performed using commercially available beads (Miltenyi Biotec, UK). Samples were processed using a 4-laser flow cytometer (LSR-Fortessa II, BD, USA) with 80,000 events captured.

5.3.2 Late-outgrowth endothelial cells (EOCs)

5.3.2.1 Isolation

Late outgrowth endothelial cells were cultured as described by Ingram *et al.* (Ingram *et al.* 2004). Mononuclear cells were isolated from peripheral blood using gradient density centrifugation with Ficoll Paque PLUS (GE healthcare, Sweden). Ten million mononuclear cells were resuspended in endothelial basal medium (EBM II Lonza, Switzerland) with 10% Hyclone fetal bovine serum (Lonza, Switzerland) and seeded onto type-1 rat's tail collagen-coated 6-well plates (BD Biosciences, USA). Cells were incubated at 37°C, 5% CO₂, 95% relative humidity. Medium was changed twice per week. Late-outgrowth colonies were counted as they emerged and cells were harvested using trypsin-EDTA for further analysis at 3-4 weeks.

5.3.2.2 Functional analysis

The migratory potential of EOCs was assessed using a standard 'scratch' assay. Cells were seeded onto type-1 rat's tail collagen-coated 6-well plates (BD Biosciences, UK) and maintained in endothelial basal medium (EBM II, Lonza, Switzerland) until confluent. A scratch was then made with a sterile pipette tip and images were acquired at 0 and 16 hours (Zeiss Observer, Carl Zeiss, Germany). Images were analysed offline using semi-automated software (Image J, NIH, Bethesda, USA). Wound healing as a measure of the migratory potential

of EOC, was expressed as percentage of the original scratch covered by 16 hours.

5.3.4 Statistical analysis

Results are expressed as mean with standard deviation or median with inter-quartile range as appropriate. Comparisons between normally distributed variables were made using paired and unpaired t-tests and ANOVA. For non-normally distributed variables, populations were compared using Mann-Whitney and Wilcoxon matched pairs tests for paired and unpaired data respectively. Kruskal Wallis tests were used for comparisons between multiple groups with non-normal distributions. Correlation was performed using Spearman or Pearson analyses as appropriate. Analyses were performed using SPSS version 21.0 (IBM Corporation, USA).

5.4 RESULTS

The demographics of the study population are outlined in **Table 1**.

Table 1: Clinical characteristics of study population (n=50)

| | Mean±SD, n (%) |
|---------------------------|----------------|
| Age | 64±10 |
| Male | 35 (70%) |
| Hypertension | 37 (74%) |
| Diabetes | 7 (14%) |
| Current smoker | 10 (20%) |
| Hypercholesterolemia | 28 (56%) |
| Family history | 38 (76%) |
| Previous radial access | 7 (14%) |
| PCI | 18 (36%) |
| Total cholesterol (mg/dL) | 176± 8.5 |
| Creatinine (mg/dL) | 0.9±0.2 |
| C-reactive protein (mg/L) | 3.7±4.4 |
| Medications | |
| Aspirin | 46 (92%) |
| Clopidogrel | 30 (60%) |
| Beta-blocker | 41 (82%) |
| ACE-I/ ARB | 18 (36%) |
| Nitrate | 10 (20%) |
| Statin | 48 (90%) |

PCI= percutaneous coronary intervention, ACE-I= angiotensin-converting enzyme inhibitor, ARB= Angiotensin receptor-blocker, OCT=optical coherence tomography

5.4.1 Endothelial Progenitor Cells and Vascular Injury

There was no change in the number of CD34⁺ cells from baseline to 24h following cardiac catheterisation irrespective of whether the procedure induced vascular injury (median [inter-quartile range] 12.0 [7.8-19.0] *versus* 15.0 [12.4-32.3] x 10⁶ cells/L, p=0.08) or not (13.0 [7.8-23.0] *versus* 16.0 [11.1-21.0] x 10⁶ cells/L, p=0.50). There was an increase in CD133⁺ cells at 24h which was statistically significant in those with evidence of vascular injury (2.3 [1.2-3.7] *versus* 2.8 [2.3-6.1] x 10⁶ cells/L, p=0.02) but not in those without (1.7 [0.7-2.5] *versus* 2.6 [1.4-4.9], p=0.09). There were no changes in CD34⁺KDR⁺ or CD34⁺133⁺KDR⁺ cells at 24h in either group (**Figure 1**). When patients were stratified by degree of vascular injury, there were no differences in CD34⁺, CD133⁺ CD34⁺KDR⁺ or CD34⁺133⁺KDR⁺ cell concentrations between groups (cellular concentrations at baseline and 24h as well as change from baseline were compared across groups; see **Tables 2-4**).

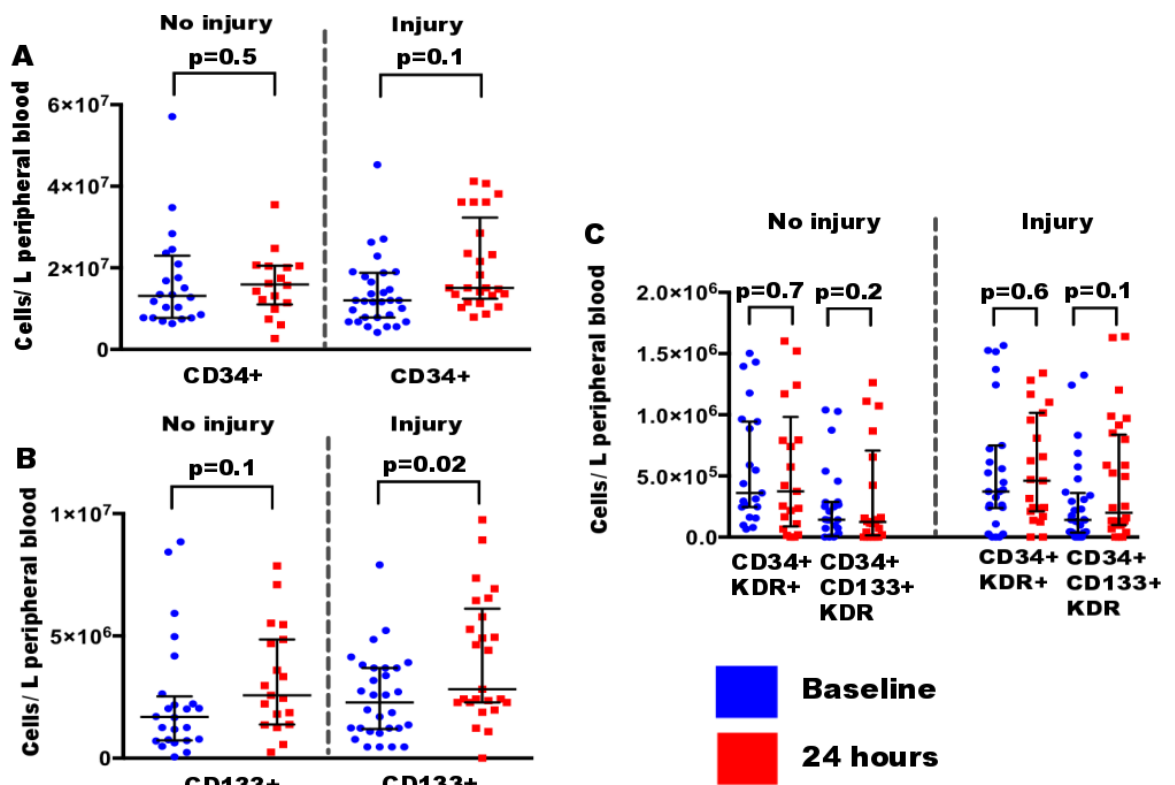


Figure 1: The effect of arterial injury on peripheral blood cellular profiles following angiography. Patients were divided into those who had sustained vascular injury (defined as having either radial artery damage seen on OCT, PCI or both) and those who had a diagnostic angiogram alone with no evidence of radial trauma. There was no significant change in peripheral blood CD34⁺ cells at 24h post-angiography in either group **(A)**. There was a modest increase in CD 133⁺ cells at 24h post-angiography which was significant in those with evidence of vascular injury but not in those without **(B)**. There was no significant increase in CD34⁺KDR⁺ or CD34⁺CD133⁺KDR⁺ cell concentration in either group **(C)**.

Values shown are median with inter-quartile range. Wilcoxon matched pairs test was used for comparisons between baseline and post-angiography samples.

| Population | No injury (n=20) | PCI only (n=15) | Radial injury (n=10) | Radial injury and PCI (n=3) | P |
|-----------------|----------------------|--------------------|-------------------------|--------------------------------|-------------|
| 45+or-ve | | | | | |
| 34+ | 13.1 (7.7–22.7) | 12.8 (7.9–18.1) | 11.8 (6.8–23.0) | 6.8 (5.6–12.0) | 0.69 |
| 133+ | 1.7 (0.7–2.6) | 2.6 (1.1–3.6) | 2.6 (1.4–3.8) | 1.2 (0.5–3.7) | 0.61 |
| 34+133+ | 1.1 (0.1–2.1) | 1.3 (0.6–3) | 1.6 (1.1–3.2) | 1.2 (0.5–3.2) | 0.97 |
| 34+KDR+ | 0.2 (0.2–0.9) | 0.4 (0.2–0.9) | 0.4 (0.2–1.3) | 0.3 (0.0–0.5) | 0.60 |
| 133+KDR+ | 0.1 (0.1–0.6) | 0.2 (0.3–0.6) | 0.2 (0.1–0.8) | 0.0 (0.0–0.8) | 0.39 |
| 34+133+KDR+ | 0.1 (0.1–0.3) | 0.1 (0.2–0.3) | 0.3 (0.0–0.6) | 0.0 (0.0–0.04) | 0.59 |
| 45- | | | | | |
| 34+ | 0.4 (0.2–1.7) | 0.6 (0.2–1.8) | 0.5 (0.3–2.8) | 0.6 (0.6–0.7) | 0.31 |
| 133+ | 0.2 (0.0–0.4) | 0.2 (0.1–0.6) | 0.3 (0.1–1.5) | 0.1 (0.0–2.3) | 0.30 |
| 34+133+ | 0.1 (0.0–0.3) | 0.1 (0.1–0.4) | 0.1 (0.1–1.2) | 0.1 (0.0–0.2) | 0.40 |
| 34+KDR+ | 0.1 (0.0–0.1) | 0.1 (0.0–0.2) | 0.1 (0.0–0.2) | 0.2 (0.0–0.2) | 0.98 |
| 133+KDR+ | 0.0 (0.0–0.1) | 0.0 (0.0–0.1) | 0.0 (0.0–0.1) | 0.0 (0.0–0.7) | 0.51 |
| 34+133+KDR+ | 0.0 (0.0–0.1) | 0.0 (0.0–0.1) | 0.0 (0.0–0.1) | 0.1 (0.0–0.2) | 0.57 |
| 45+ | | | | | |
| 34+ | 13.7 (7.1–23.0) | 12.6 (4.2–20.0) | 11.1 (4.1–18.5) | 6.5 (1.3–11.8) | 0.29 |
| 133+ | 2.6 (1.3–6.5) | 3.8 (1.8–5.3) | 2.4 (1.4–5.6) | 1.3 (0.5–2.4) | 0.45 |
| 34+133+ | 1.2 (0.9–3.8) | 1.9 (0.7–3.4) | 1.3 (1.1–3.4) | 1.1 (0.5–2.1) | 0.75 |
| 34+KDR+ | 1.4 (0.6–2.9) | 0.5 (0.3–1) | 0.4 (0.2–0.9) | 0.1 (0.1–0.4) | 0.02 |
| 133+KDR+ | 0.3 (0.1–0.6) | 0.4 (0.2–1.4) | 0.6 (0.1–0.8) | 0.1 (0.1–0.2) | 0.39 |
| 34+133+KDR+ | 0.2 (0.1–0.6) | 0.2 (0.2–0.4) | 0.3 (0.1–0.6) | 0.1 (0.1–0.2) | 0.69 |

Table 2:

Absolute cell numbers in peripheral blood at baseline prior to angiography. Cell numbers are expressed in numbers of cells per litre of peripheral blood. Krusal-Wallis tests were used to compare cell numbers between injury groups.

| Population | No injury (n=20) | PCI only (n=15) | Radial injury (n=10) | Radial injury and PCI (n=3) | P |
|-----------------|------------------------|------------------------|-------------------------|--------------------------------|-------------|
| 45+or-ve | | | | | |
| 34+ | 16.6 (12.0 – 22.2) | 15.1 (1.3 – 23.5) | 13.2 (9.5 – 30.0) | 15.1 (5.3 – 36) | 0.71 |
| 133+ | 2.6 (1.4 – 4.9) | 4.4 (2.0 – 6.5) | 3.9 (2.0 – 7.8) | 2.3 (1.0 – 2.4) | 0.44 |
| 34+133+ | 1.6 (0.5 – 3.5) | 2.0 (1.3 – 4.7) | 2.0 (1.3 – 4.8) | 2.0 (1.0 – 2.0) | 0.36 |
| 34+KDR+ | 0.4 (0.1 – 1.1) | 0.8 (0.2 – 1.2) | 0.4 (0.2 – 1.3) | 0.2 (0.0 – 0.2) | 0.15 |
| 133+KDR+ | 0.2 (0.0 – 0.8) | 0.6 (0.1 – 1.0) | 0.4 (0.1 – 1.6) | 0.1 (0.0 – 0.2) | 0.49 |
| 34+133+KDR+ | 0.1 (0.0 – 0.6) | 0.5 (0.1 – 0.9) | 0.3 (0.1 – 1.0) | 0.1 (0.0 – 0.1) | 0.73 |
| 45- | | | | | |
| 34+ | 0.5 (0.1 – 0.8) | 0.4 (0.1 – 1.2) | 0.3 (0.1 – 0.7) | 0.3 (0.0 – 0.5) | 0.72 |
| 133+ | 0.1 (0.0 – 0.3) | 0.1 (0.0 – 0.3) | 0.2 (0.1 – 0.9) | 0.0 (0.0 – 0.1) | 0.22 |
| 34+133+ | 0.1 (0.0 – 0.2) | 0.1 (0.0 – 0.2) | 0.1 (0.0 – 0.6) | 0.0 (0.0 – 0.1) | 0.16 |
| 34+KDR+ | 0.1 (0.0 – 0.1) | 0.1 (0.0 – 0.1) | 0.1 (0.0 – 0.2) | 0.0 (0.0 – 0.0) | 0.70 |
| 133+KDR+ | 0.0 (0.0 – 0.1) | 0.0 (0.0 – 0.0) | 0.0 (0.0 – 0.2) | 0.0 (0.0 – 0.0) | 0.43 |
| 34+133+KDR+ | 0.0 (0.0 – 0.0) | 0.0 (0.0 – 0.0) | 0.0 (0.0 – 0.1) | 0.1 (0.0 – 0.1) | 0.74 |
| 45+ | | | | | |
| 34+ | 15.3 (9.9 – 20.0) | 15.7 (12.2 – 26.4) | 11.1 (4.8 – 21.7) | 14.8 (10.0– 36.0) | 0.78 |
| 133+ | 2.7 (1.6 – 5.3) | 4.3 (2.1 – 6.2) | 2.5 (2.1 – 6.0) | 2.6 (1.3 – 2.4) | 0.28 |
| 34+133+ | 1.9 (0.8 – 3.3) | 2.0 (1.2 – 4.6) | 1.9 (1.2 – 3.8) | 1.9 (1.9 – 4.1) | 0.53 |
| 34+KDR+ | 0.9 (0.6 – 2.1) | 0.8 (0.2 – 1.1) | 0.3 (0.2 – 0.9) | 0.2 (0.2 – 0.2) | 0.04 |
| 133+KDR+ | 0.2 (0.1 – 0.8) | 0.6 (0.1 – 1.0) | 0.5 (0.1 – 1.5) | 0.1 (0.1 – 0.2) | 0.62 |
| 34+133+KDR+ | 0.1 (0.1 – 0.7) | 0.5 (0.1 – 0.8) | 0.5 (0.1 – 0.8) | 0.1 (0.0 – 0.11) | 0.65 |

Table 3: Absolute cell numbers in peripheral blood at 24h post angiography. Cell numbers are expressed in numbers of cells per litre of peripheral blood. Krusal-Wallis tests were used to compare cell numbers between injury groups.

| Population | No injury (n=20) | PCI only (n=15) | Radial injury (n=10) | Radial injury and PCI (n=3) | P |
|-----------------|---------------------|--------------------|-------------------------|--------------------------------|------|
| 45+or-ve | | | | | |
| 34+ | +2.9 (-8.4 - 11.9) | +6.6 (-1.8 - 10.5) | +1.6 (-4.0 - 5.5) | +8.3 (7.2 - 24.1) | 0.81 |
| 133+ | +1.2 (-0.5 - 3.0) | +1.6 (-0.6 - 3.8) | +1.3 (-0.5 - 8.0) | -1.1 (-1.4 - 1.2) | 0.67 |
| 34+133+ | +0.4 (-0.4 - 1.9) | +1.0 (-0.4 - 0.5) | +0.9 (-0.0 - 2.3) | -0.8 (-1.3 - 0.7) | 0.28 |
| 34+KDR+ | -0.1 (-0.2 - 0.4) | -0.0 (-0.4 - 0.5) | +0.2 (0.0 - 0.6) | -0.2 (-0.2 - 0.6) | 0.63 |
| 133+KDR+ | +0.1 (-0.1 - 0.4) | 0.0 (-0.2 - 0.3) | +0.2 (0.0 - 0.4) | 0.0 (0.0 - 0.5) | 0.22 |
| 34+133+KDR+ | +0.1 (-0.9 - 0.2) | +0.1 (-0.2 - 0.4) | +0.1 (-0.5 - 0.6) | +0.1 (0.1 - 0.9) | 0.76 |
| 45- | | | | | |
| 34+ | +0.1 (0.2 - 0.4) | -0.1 (-0.1 - 0.4) | -0.2 (-1.8 - -0.1) | -0.4 (-0.6 - -0.2) | 0.29 |
| 133+ | -0.1 (-0.1 - 0.2) | -0.1 (-0.7 - -0.1) | 0.0 (-0.1 - 0.2) | -0.1 (-2.3 - 0.2) | 0.08 |
| 34+133+ | 0.0 (-0.2 - 0.1) | -0.1 (-0.4 - 0.0) | 0.0 (-0.1 - 0.1) | -0.2 (-0.2 - 0.0) | 0.31 |
| 34+KDR+ | 0.0 (-0.1 - 0.1) | -0.0 (-0.1 - 0.0) | 0.0 (-0.1 - 0.1) | 0.0 (-0.1 - 0.1) | 0.44 |
| 133+KDR+ | 0.0 (-0.0 - 0.0) | -0.0 (-0.1 - 0.0) | 0.0 (0.0 - 0.1) | 0.0 (-0.0 - 0.0) | 0.12 |
| 34+133+KDR+ | 0.0 (-0.0 - 0.0) | -0.0 (-0.1 - 0.0) | 0.0 (0.0 - 0.0) | 0.0 (-0.0 - 0.0) | 0.32 |
| 45+ | | | | | |
| 34+ | +3.2 (-9.1 - 13.6) | +2.5 (-4.1 - 18.5) | +0.9 (-8.0 - 2.1) | -1.8 (-6.5 - 2.9) | 0.74 |
| 133+ | -0.2 (-1.9 - 0.9) | -0.4 (-1.0 - 0.6) | +0.1 (-0.9 - 0.9) | -0.1 (-0.5 - 1.0) | 0.62 |
| 34+133+ | +0.1 (-1.2 - 0.9) | +0.2 (-0.8 - 1.8) | +0.6 (-0.6 - 0.7) | +0.3 (-0.2 - 0.8) | 0.39 |
| 34+KDR+ | -0.4 (-0.7 - 0.2) | -0.0 (-0.3 - 0.5) | +0.0 (-0.2 - 0.3) | -0.1 (-0.2 - 0.1) | 0.27 |
| 133+KDR+ | 0.0 (-0.2 - 0.1) | -0.2 (-0.4 - 0.1) | +0.1 (-0.0 - 0.5) | 0.0 (0.0 - 0.1) | 0.14 |
| 34+133+KDR+ | 0.0 (-0.2 - 0.1) | -0.5 (-0.6 - 0.3) | -0.1 (-0.1 - 0.2) | -0.1 (-0.1 - 0.1) | 0.98 |

Table4: Absolute change in cell numbers in peripheral blood at from baseline to 24 hrs post-angiography. Cell numbers are expressed in numbers of cells per litre of peripheral blood. Krusal-Wallis tests were used to compare cell numbers between injury groups.

5.4.2 Late outgrowth endothelial cells

Isolated EOCs had a typical ‘cobblestone’ monolayer morphology and consistently expressed CD31 and CD34 (**Figure 2**). Compared to baseline, there was no difference in the number of EOC colonies isolated at 24h (1.0 [0.0-3.0] *versus* 0.5 [0.0-2.0] colonies *per patient*, $p=0.06$). There was no difference in the migratory function of EOCs isolated at baseline and 24h ($50.0\pm2.9\%$ *versus* $49.3\pm2.9\%$ wound coverage, $p=0.82$; **Figure 2**). Compared to colonies isolated at baseline, there were no changes in EOC expression of CD34 ($69.7\pm9.1\%$ *versus* $67.8\pm9.9\%$, $p=0.80$), CD31 ($94.1\pm2.8\%$ *versus* $98.3\pm0.4\%$, $p=0.61$), KDR ($61.0\pm5.3\%$ *versus* $58.0\pm8.0\%$ respectively, $p=0.85$) or CD146 ($95.4\pm2.3\%$ *versus* $98.5\pm0.5\%$ respectively, $p=0.42$) in those colonies isolated at 24h (**Figure 2**).

5.4.3 Cellular predictors of baseline arterial function and recovery

Baseline endothelial function (defined as FMD in the right radial artery) was negatively correlated with baseline CD34⁺ ($r=-0.4$, $p=0.004$) and CD133⁺ ($r=-0.33$, $p=0.02$) cell concentrations. Recovery of arterial function (defined as the recovery by 3 months of the deficit seen at 24h) was negatively correlated with baseline CD34⁺ concentration ($r=-0.33$, $p=0.04$). Neither baseline arterial function nor recovery post-injury correlated with the number of late outgrowth endothelial cell colonies isolated. There was a positive correlation between the

migratory capacity of EOC, and baseline endothelial function ($r=0.47$, $p=0.03$), but not recovery following injury ($r=0.02$, $p=0.94$; **Table 5**).

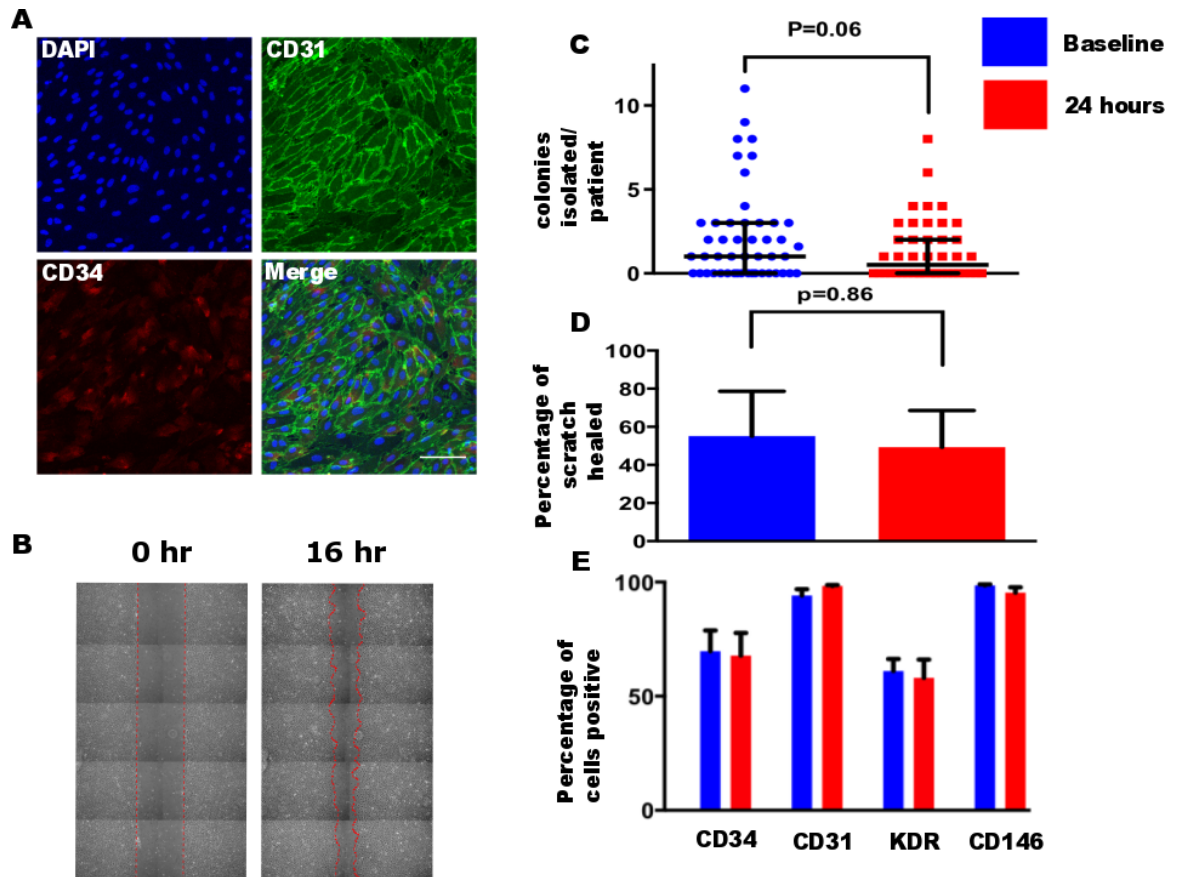


Figure 2: Late-outgrowth endothelial colony and wound healing analysis
Colonies of late-outgrowth endothelial cells were isolated and characterised. Immunofluorescence for DAPI (nuclei, blue), CD31 (FITC; green) and CD34 (Alexa 568; red) was performed. Cells had a comparable phenotype to vascular endothelial cells with ubiquitous expression of CD31 and CD34. Scale bars: 100 μ m. **(A)** Assessment of EOC migratory capacity was assessed using a 'scratch' wound healing assay, between 0 and 16h **(B)** There was no significant difference in the number of EOC colonies isolated **(C)**, their migratory potential **(D)**, or cell-surface marker profile ($p>0.05$ for all comparisons) **(E)**, between colonies isolated at baseline and 24h post-angiography.

| Baseline arterial function | <u>R</u> | <u>P</u> |
|--------------------------------------|-----------------|-----------------|
| <u>Correlate</u> | | |
| CD34+ | -0.40 | 0.004 |
| CD34+CD133+ | -0.13 | 0.37 |
| CD34+KDR+ | -0.24 | 0.10 |
| CD34+CD133+KDR+ | -0.27 | 0.08 |
| CD133+ | -0.33 | 0.02 |
| CD34+CD45- | -0.17 | 0.23 |
| Mean colony number | 0.01 | 0.92 |
| Wound healing | 0.47 | 0.03 |
| Recovery of arterial function | | |
| <u>Correlate</u> | <u>R</u> | <u>P</u> |
| CD34+ | -0.33 | 0.04 |
| CD34+CD133+ | -0.29 | 0.06 |
| CD34+KDR+ | -0.08 | 0.60 |
| CD34+CD133+KDR+ | -0.02 | 0.90 |
| CD133+ | -0.19 | 0.20 |
| CD34+CD45- | -0.02 | 0.49 |
| Mean colony number | -0.14 | 0.38 |
| Wound healing | 0.02 | 0.94 |

Table 5: Cellular predictors of baseline endothelial function and recovery following injury. Baseline arterial function then subsequently recovery of function following injury was correlated with various cellular populations as well as the function of isolated endothelial outgrowth cells. Cellular populations were expressed as absolute cell numbers/L of peripheral blood and wound healing was expressed as the percentage of a scratch healed by 16h. Baseline endothelial function was defined as left radial artery flow-mediated dilatation pre-angiography and recovery of arterial function was defined as the percentage recovery by three months of the deficit observed at 24 h in the catheterised artery

5.5 DISCUSSION

Circulating concentrations of EPCs (defined by cell surface antigen phenotype) have been correlated with improved vascular function and reduced cardiovascular events (Werner *et al.* 2005; Liao *et al.* 2010; Hill *et al.* 2003). However, controversy surrounds the correct definition of a *bona fide* EPC (Yoder 2009; Padfield *et al.* 2013). We observed an increase in CD133⁺ and CD34⁺CD133⁺ cells at 24h although we found no correlation with either the extent of injury or recovery of vasomotor function suggesting this increase may simply represent a non-specific reaction to an inflammatory insult rather than a discrete EPC mobilisation. Whilst EPCs may exist as sub-populations of CD34⁺ cells, CD133 and CD34 expressing cells represent a naïve, heterogeneous population including haematopoietic cells with non-specific roles in tissue repair (Jing Zhao *et al.* 2014; Wognum *et al.* 2003) and elevated concentrations likely reflect vascular inflammation and cellular turnover. In support of this, we observed a negative correlation between CD34 and CD133 positive cells and baseline endothelial function.

Mobilisation of CD34⁺ and CD133⁺ cells has previously been reported in response to large non-specific inflammatory insults (Gao *et al.* 2015; Thomas *et al.* 2008; Inoue *et al.* 2007) and myocardial infarction (Schober *et al.* 2005; Padfield *et al.* 2013) whereas cellular response to discrete vascular injury is less readily demonstrated (Padfield *et al.* 2014; Gao *et al.* 2015; Thomas *et al.* 2008). Acute mobilisation of CD34⁺ populations has also been described post-PCI with

higher concentrations predicting restenosis (Inoue *et al.* 2007; Schober *et al.* 2005). Alongside the disappointing experience of CD34⁺-capture stents (Klomp *et al.* 2011), this supports a non-specific inflammatory role for these cells post-injury.

Despite an increase in circulating CD133⁺ cells at 24h, we observed no difference in rates of EOC colony isolation, supportive of previous work suggesting that EOCs, the presumed progeny of circulating EPC, cannot be isolated from the CD133⁺ fraction of peripheral blood (Timmermans *et al.* 2007; Tura *et al.* 2013).

We demonstrated a positive correlation between EOC migratory capacity and baseline endothelial function, although EOC migration did not predict recovery post-injury. Consistent with previous work in uraemia (Zhao *et al.* 2014), obesity (Richards *et al.* 2014) and following *in vitro* exposure to oxidised LDL (Lin *et al.* 2015), our finding of a positive correlation between EOC migratory activity and baseline endothelial function raises the possibility that characterisation of EOC function may afford a more accurate picture of vascular health than surface marker-defined peripheral blood EPCs. Late outgrowth endothelial cells are thought to play a central role in vascular regeneration (Ingram *et al.* 2004; Yoder 2009). In contrast to early outgrowth endothelial and CD34⁺ cells, late outgrowth endothelial cells are capable of incorporating into native vessels following transplantation in animal models of angiogenesis, increasing capillary density and reducing neo-intimal hyperplasia (Barclay *et al.*

2012; Minami *et al.* 2015; Schwarz *et al.* 2012). Given these regenerative capabilities, it is perhaps not surprising that late outgrowth endothelial cell function *in vitro* correlates with radial artery vasomotion.

5.6 CONCLUSION

This study characterised the cellular profile which accompanies radial artery catheterisation and examines possible modifying effects of these populations on vessel recovery. Heterogeneous populations of circulating cells such as those defined by markers of cellular naïveté such as CD133 or CD34 do not seem to enhance vascular repair but late-outgrowth endothelial cell function correlates with radial vasomotor function at baseline, underlining the importance of this cell population to vascular health.

Chapter 6

**GMP-compliant culture of a stable and phenotypically
homogenous late outgrowth endothelial cell product
for human administration**

SUMMARY

Despite the intense interest in regenerative medicine for cardiovascular diseases, trials of cellular therapies have been largely disappointing and this may be a reflection of the cell substrates employed. Late outgrowth endothelial cells (EOCs) are highly proliferative cells of endothelial lineage which contribute to repair in animal models of vascular injury and may be useful as a regenerative therapy in cardiovascular medicine.

Our laboratory's well-established research-grade EOC culture protocol was translated by sequential substitution of animal-origin components for GMP compliance. Cells produced using this GMP compliant protocol were then characterised in terms of gross morphology and cell surface marker profile to confirm an EOC phenotype. In addition, viability and stability of cell surface marker expression was assessed out to 24h.

Using a fully GMP-compliant culture protocol we were able to isolate and expand EOCs from all donors (n=4, mean EOC yield $8.9 \pm 12.1 \times 10^6$ cells per patient). Cells had high CD31 expression ($98.1 \pm 1.5\%$), low CD45 expression ($1.9 \pm 1.5\%$) and high viability ($87.6 \pm 4.3\%$). EOC viability remained high out to 6h ($82.7 \pm 2.0\%$) but had fallen significantly by 24h (43 ± 18.6 p=0.02) when compared to baseline.

We describe the translation of a research-grade to GMP-compliant culture protocol which reliably yields phenotypically consistent EOCs and will allow trials of human EOC administration.

6.1 INTRODUCTION

Since the first human trials over a decade ago (Strauer 2002; Assmus *et al.* 2002), there has been intense interest in cellular therapies for cardiovascular disease. Myocardial regeneration has been the main focus of this activity in both ischaemic and non-ischaemic heart failure. However, recent large meta-analyses point to the fact that trials of these therapies in heart failure are in general characterised by modest treatment effects and lack of adequate control arms (Fisher *et al.* 2015). Indeed a recent meta-analysis found that treatment effects observed in cardiovascular cell therapy trials are directly proportional to the potential bias present in the trial design (Nowbar *et al.* 2014).

There has been similar interest in cell therapies for vascular regeneration in peripheral vascular disease with a recent well conducted meta-analysis suggesting benefit in terms of amputation-free survival (Rigato *et al.* 2017). As with cell therapies for myocardial regeneration, high-quality randomised controlled trials were in the minority in this meta-analysis, limiting the validity of the conclusions drawn.

To date, the majority of cell therapy trials have administered bone marrow aspirate or mobilised peripheral blood with the only refinement being selection for a non-specific marker of such as CD34 or CD133 (Fisher *et al* 2015). Culture-modified cells have been employed as a regenerative therapy in ischaemic cardiomyopathy (Makkar *et al.* 2012), and peripheral vascular disease (Szabo *et al.* 2013). In myocardial regeneration, the main cell of interest has been the cardiosphere, isolated from myocardial biopsy samples and expanded in culture. Whilst initial clinical trial experience has been positive (Makkar *et al.* 2012), there is some doubt as to whether these cells can differentiate into mature myocardium, either *in vitro* (Andersen *et al.* 2009) or *in vivo* (Shenje *et al.* 2008). The cells used in peripheral vascular disease (Szabo *et al.* 2013) resemble previously described early outgrowth endothelial cells (Hill *et al.* 2003) which do not directly contribute to endothelial regeneration (Yoder 2009). The use of partially-differentiated cells for cardiovascular regeneration has therefore not been fully explored.

Late-outgrowth endothelial cells (EOCs) are thought to arise from a circulating endothelial progenitor cell, and it has been proposed that EOCs have the capacity to home to and proliferate at sites of vascular injury, reconstituting damaged endothelium. They incorporate into damaged vessel walls and increase capillary density in experimental models of limb ischaemia (Barclay *et al.* 2012; Minami *et al.* 2015; Schwarz *et al.* 2012). These cells can be isolated from unmobilised peripheral blood, and the dysfunctional phenotype seen in other cell lines such as early outgrowth endothelial cells (Hill *et al.* 2003)

isolated from patients with cardiovascular disease does not seem to exist for EOCs (Dauwe *et al.* 2016). These observations make EOCs a promising candidate for use as a cellular therapy.

Whilst methods for the culture of these cells are well described (Ingram *et al.* 2004), neither GMP-compliant production nor human administration have been attempted. The objective of this study was to translate a well-established preclinical EOC culture protocol into a GMP compliant process for use in humans with detailed characterisation of the resultant cell product.

6.2 METHODS

6.2.1 Subjects

Subjects were recruited from the waiting list for elective outpatient coronary angiography for known or suspected coronary artery disease at the Royal Infirmary of Edinburgh, UK. Patients experiencing a recent acute coronary syndrome or recent cardiac catheterisation (<3 months), severe valvular heart disease, renal impairment (estimated glomerular filtration rate <30 mL/min), contrast allergy, pregnancy, inability to give informed consent and those aged below 18 or above 85 years were excluded. The study was approved by the local research ethics committee and written informed consent was obtained from all patients.

6.2.2 Research-grade EOC isolation protocol

Peripheral blood (100-200mL) was collected in 50 mL centrifuge tubes (Falcon™, Corning, US) with 3.8% sodium citrate buffer from an antecubital vein using an 18-gauge cannula. Endothelial outgrowth cells (EOC) were then isolated using a protocol modified from Ingram *et al* (Ingram *et al.* 2004). Briefly, mononuclear cells were separated from peripheral blood using gradient-density centrifugation with Ficoll Paque PLUS (GE healthcare, Sweden). Mononuclear cells were suspended in endothelial growth medium (EBM II Lonza, Switzerland) with 10% Hyclone foetal bovine serum (Lonza, Switzerland) and seeded onto type-1 rat tail collagen-coated T75 flasks (BD Biosciences, US). Cells were incubated at 37°C, 5% CO₂, 95% relative humidity. The floating cells were removed 24h later and fresh medium was added to each well following PBS wash. Medium was replaced twice per week. Cells were grown for 3 weeks and colony numbers were counted before harvesting with TrypLE express (Thermo Fisher Scientific, UK). Cells were subsequently cultured in type-1 rat tail collagen-coated T75 flasks (BD Biosciences, USA).

6.2.3 Translation of research to GMP-compliant culture

Two crucial base materials in our laboratory's EOC culture protocol are rat tail collagen I which coats the plastic cultureware, and foetal bovine serum which is added to the basal culture medium which acts as a protein supplement. These are both of animal origin and for the purposes of GMP-compliant protocol

development we tested xeno-free alternatives. We assessed the efficacy of the various substitute materials with their ability to support established EOC populations and to support EOC colony isolation being assessed. Following this, EOC manufacture was carried out in a clean room environment under full GMP conditions (**Figure 1**). Our final GMP-compliant protocol is outlined below and substitute materials tested in early iterations of the protocol are outlined in (**Table1**).

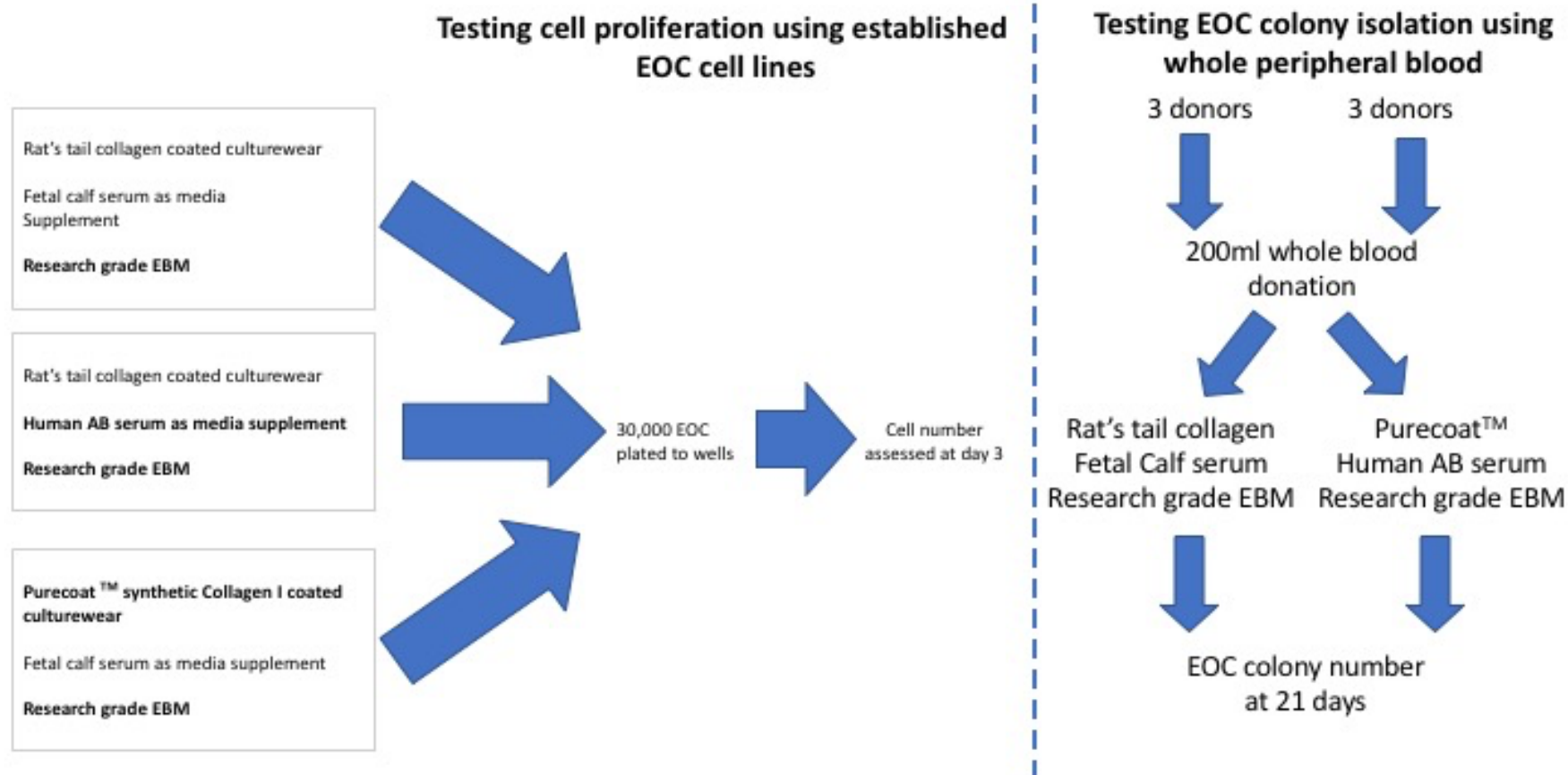


Figure 1: Testing GMP-compliant substitution

Table 1: Translation of research grade EOC culture process

| Component of culture process | Research protocol materials | Substitutions unsuccessfully trialed | Final GMP protocol |
|--------------------------------------|---|---|--|
| Mononuclear cell separation | FICOLL separation (Ficollpaque Plus TM , GE, US) | No additional substitutions trialed | GMP Grade FICOLL (FiccollPaquePREMIUM TM , GE, US) |
| | | | Leucosept tubes (Sigma-Aldrich, US) |
| Attachment media | Rat-tail Collagen type I | Cellstart TM (Thermofisher, US) | Purecoat TM synthetic Collagen I (Corning, US) |
| Culture medium and supplement | EGM-2 (Lonza) 10% Hyclone FBS | Unsupplemented media | GMP-grade customized EBM-2 (Lonza, US) media with EGM-2 Singlequot kit |
| | | Autologous serum | Without heparin |
| | | Human albumin serum (Sigma-Aldrich, US) | With clinical grade insulin (Lilly, UK) |
| | | Xerum-Free TM (Funakoshi, Japan) | 10% AB donor serum (SNBTS, UK) |

6.2.3 Testing substitute components for GMP-compliant culture

Xenoprotein-free attachment media and media supplements were tested to ensure equivalent efficacy to research-grade materials for EOC culture. Initially the ability of substitute materials to maintain established cell lines in culture was tested as below.

EOCs were plated at 3×10^4 per well (6-well plates) in the following conditions; rat tail collagen I coated plate with Hyclone FCS supplemented medium, rat tail collagen I coated plate with human AB donor serum-supplemented media, or Purecoat™ synthetic collagen I plate with Hyclone FCS-supplemented media. The media was changed every 24 h and wells were captured at 3 days using a live cell imaging device with x4 objective lens (incucyte®, Essen Bioscience, USA). The number of cells per field was counted. Cells from three separate donors were used for each experiment with two technical repeats.

Once efficacy of these substitute materials in maintaining EOC growth was confirmed, three donors were recruited and EOC isolation was carried-out (as described below) using research-grade basal media, AB donor serum (Sigma-Aldrich) and Purecoat™ synthetic Collagen I (Corning, USA) tissue culture. Efficacy was compared to six donors in whom isolation was performed with standard research-grade materials.

6.2.4 EOC isolation and expansion under GMP conditions

Once our culture media substitutes had been shown to be effective in supporting EOC isolation and expansion, four process validation cultures were carried out, isolating and expanding EOC from four donors under full GMP conditions using a custom-manufactured endothelial basal media (EBM-2 with EBM-2 SingleQuot kit, custom-manufactured in GMP facility, Lonza, US) as described below. The cells produced were subject to detailed phenotypic analysis at the end of the culture process.

Peripheral blood (200-250 mL) was taken from a large antecubital vein using a 16-gauge needle and dedicated venesection bag containing sodium citrate (Macropharma, Phillipines). Endothelial outgrowth colonies were isolated under GMP conditions in dedicated clean rooms. Briefly, mononuclear cells were separated from unmobilised peripheral blood using density centrifugation with Leucosep™ tubes (Greiner Bio-One, Germany) and Ficoll-paque PREMIUM (GE, USA). All mononuclear cells obtained were then re-suspended in GMP-grade endothelial growth medium (EBM-2 with EBM-2 SingleQuot kit, custom-manufactured in GMP facility, Lonza, US). Heparin was not added to the media and the insulin supplied by the manufacturers was replaced with clinical grade insulin (20ng/mL, Humulin S, Eli Lilly, UK). Media was supplemented with single donor AB serum (Scottish National Blood Transfusion Service) and plated to Purecoat™ ECM Mimetic Collagen I peptide T75 flasks (Corning, USA). Non-

adherent cells were removed at 24 hours. Medium was changed twice weekly. Colonies were counted and passaged to T175 flasks on day 20-21 using TrypLE™ Select (Thermo Fisher Scientific, US) and maintained in culture until day 30.

6.2.5 Analysis of GMP EOC product

6.2.5.1 Characterisation of cell product at point of release

At the end of the culture period (culture day 30), cells were dissociated using TrypLE™ Select (Thermo Fisher Scientific) and harvested in excipient (0.5% human albumin serum (Alburex, CSL Behring, US) in 0.9% saline (Baxter, US). Samples were then analysed for phenotype, viability and sterility as outlined below.

6.2.5.2 Phenotypic analysis and viability

In addition to assessment of typical morphological appearance by microscopy at the end of the culture process, EOCs were analysed for cell surface markers using flow cytometry. Cells were washed with PBS containing EDTA (2.5 mM) and human serum albumin (0.5%, Alburex, CSL behring, US) and labelled with a panel of antibodies including CD45-VioBlue (Miltenyi, Germany), CD31-FITC (BD Biosciences, US), and DRAQ7 dead cell discriminator (Biolegend, UK). Based on previous experience of EOC culture we set release criteria which the cell

product will need to satisfy for human administration, namely >90% CD31+, < 10% CD45+ cells, and >50% viability (DRAQ7 negative).

Viability and stability of cell surface marker expression was assessed to 24 hours with the cells suspended in 0.5% human albumin serum (Alburex, CSL Behring, US) in 0.9% saline (Baxter, US) and held at 37°C with agitation. In addition to the markers described above the following were assessed: CD146-APC (Miltenyi, Germany) CD309-PE (Biolegend), CD144-PE Vio770 (Miltenyi, Germany), CD34-APC (Biolegend, UK) and DRAQ7. All samples were analysed on a BD FACSCANTO II flow cytometer (BD Biosciences, US) and mean fluorescence intensity (MFI) was used to denote cell surface marker expression.

6.2.5.3 Stability

To define the stability of the EOC product, viability and cell surface expression of CD31, CD144, CD146, CD 309 and CD34 were assessed as detailed above at baseline, 4, 6 and 24h following harvest,

6.2.5.4 Sterility testing

Sterility of the cell product was confirmed in the cells manufactured within the GMP cleanroom by inoculation of BacT/ALERT™ (Biomérieux, France) aerobic and anaerobic culture bottles with spent media harvested at selected media

changes and with the cell product on day 30. Broths were incubated and monitored for sterility over a 14-day incubation period.

6.3 RESULTS

6.3.1 Subjects

All patients were drawn from the same cohort and baseline characteristics are outlined in **Table 2**.

Table 2: Baseline characteristics.

| | <u>Process development</u> | | Process validation within clean room environment |
|----------------------------------|-----------------------------------|--------------------------------|---|
| | Research-grade materials | GMP-compliant materials | |
| | N= 6 | N=3 | N=4 |
| Age | 61±3 | 58±5 | 63±8 |
| Male | 4 (67%) | 2(67%) | 3(75%) |
| Coronary artery disease | 3 (50%) | 2(67%) | 2 (50%) |
| Hypertension | 5 (83%) | 2 (67%) | 3 (75%) |
| Diabetes | 4 (67%) | 2 (67%) | 3 (75%) |
| Current smoker | 1 (17%) | 0 (0%) | 0 (0%) |
| Family history | 3 (50%) | 1 (33%) | 1(25%) |
| Total cholesterol (mg/dL) | 138 ±9.5 | 140 ±10.1 | 150 ±25.1 |
| Creatinine (mg/dL) | 0.8 ±1.1 | 0.7 ±1.3 | 0.9 ±1.2 |

6.3.1 Culture component substitutions

Culture media without serum supplementation was not able to support EOC proliferation nor was culture-ware coated with Cellstart™ (Thermofisher, US) and these were not tested further. Both Human AB serum (Sigma Aldrich, USA) and Purecoat™ ECM Mimetic Collagen I peptide (Corning, USA) were able to support EOC proliferation when substituted into the culture protocol.

There was no difference in proliferation between EOCs cultured using our established research-grade materials (Rat tail collagen I and fetal bovine serum, 83.45 ± 13.18 cells per high-powered field) and those with AB serum (89.22 ± 42.45 cells per high-powered field) or Purecoat™ (81.87 ± 19.89 cells per high-powered field) substituted ($n=3$, $p=0.94$, ANOVA with Tukey multiple comparisons **Figure 2**).

6.3.2 Testing GMP-compliant substitutions for EOC isolation

EOC colonies were isolated from all three donors using our protocol which included human AB serum (Sigma- Aldrich) and Purecoat™ in place of fetal bovine serum and rat tail collagen I respectively. Mean colony number was 23.50 ± 18.26 colonies/100mL blood similar to the yield obtained using standard research materials in six separate donors (19.50 ± 10.89 colonies/100mL blood, $p=0.65$; **Figure 3**).

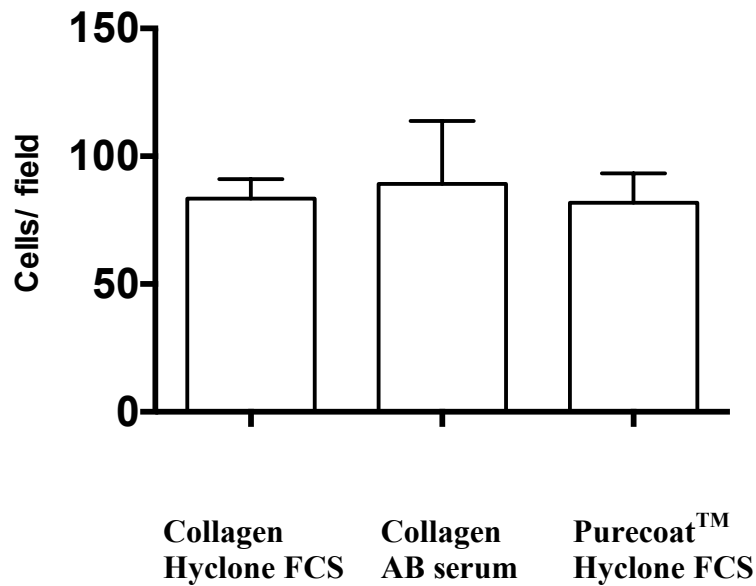


Figure 2: Testing substitution of Purecoat™ and human AB serum against research-grade materials. There was no difference in proliferation between EOCs cultured using our established research-grade materials (Rat tail collagen I and Hyclone fetal calf serum,) and those with human AB serum or Purecoat™ substituted ($p=0.96$, ANOVA with Tukey multiple comparisons). $N=3$ with 2 technical repeats.

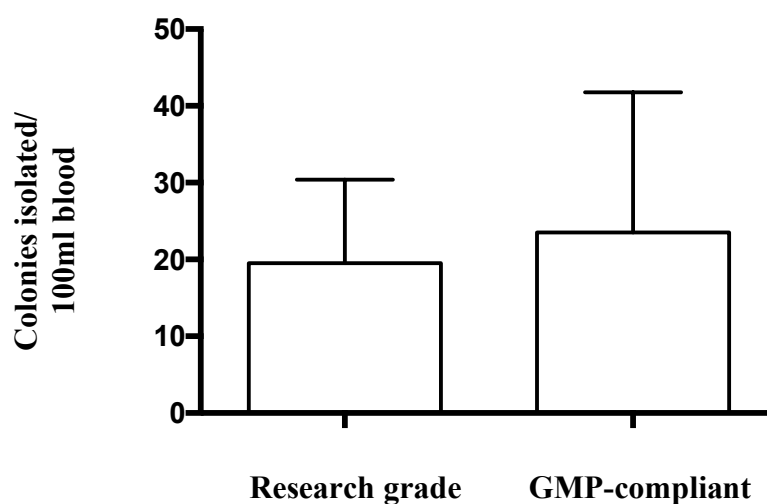


Figure 3: Colony yield using GMP-compliant substitute materials compared to research-grade materials. EOC colonies were isolated from all donors. There was no difference in colony yield between GMP-compliant culture materials (Purecoat™ and human AB serum) 23.50 ± 18.26 and standard research-grade materials (rat tail collagen I and Hyclone fetal calf serum) 19.50 ± 10.89 , $p = 0.65$ (two tailed unpaired t test). $N=3$ for GMP-compliant materials and $N=6$ for research-grade materials.

6.3.3 Colony isolation using GMP-compliant culture protocol

Late outgrowth colonies were isolated in all cases (mean 6.5 ± 2.4 colonies per patient). The mean EOC yield at the end of the culture process was $8.9 \pm 12.1 \times 10^6$ cells per patient.

6.3.4 Characterisation of cells produced under GMP conditions.

All cells produced using the GMP protocol met pre-defined release criteria with high CD 31 expression ($98.1 \pm 1.5\%$), low CD45 expression ($1.9 \pm 1.5\%$) and high viability ($87.6 \pm 4.3\%$; **Figure 4**).

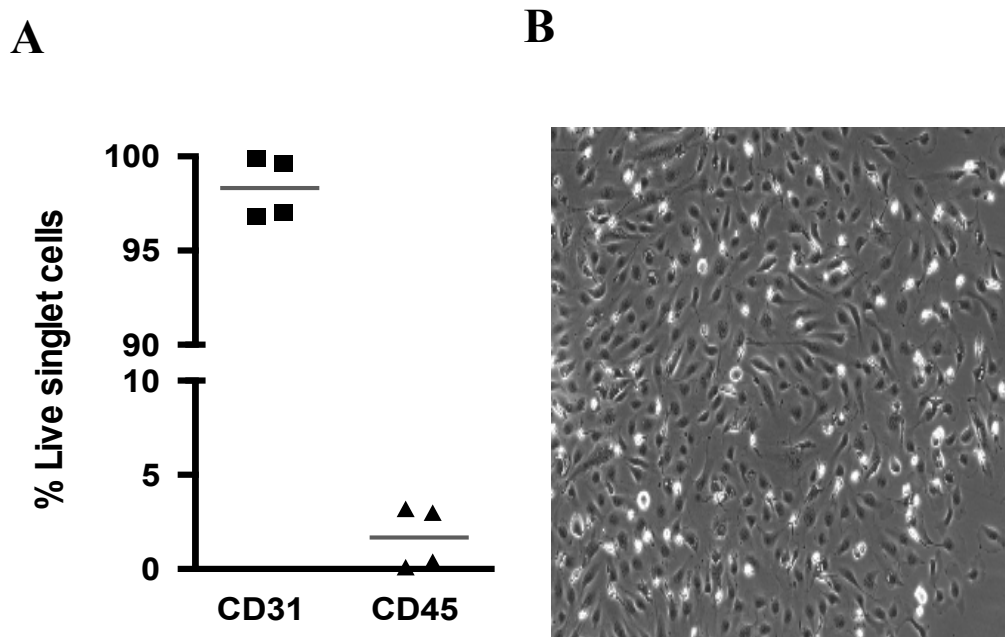


Figure 4: Purity of process validation cultures.

A. EOC cultures from four donors were phenotypically consistent with high CD31 purity ($98.1 \pm 1.5\%$) and low CD45+ cell contamination ($1.9 \pm 1.5\%$). Results were used to establish the release criteria for suitable cell products as a CD31 purity of $\geq 90\%$ and CD45 contamination of $\leq 10\%$. N=4. **B.** Typical endothelial morphology with cobblestone monolayer.

6.3.5 Stability of GMP EOCs

Following harvest within the GMP facility and subsequent storage in excipient at 37°C, viability remained high at 4h ($87.3 \pm 4.6\%$, $p > 0.05$) and 6h ($82.7 \pm 2.0\%$, $p > 0.05$) but fell significantly by 24h (43 ± 18.6 $p = 0.02$, ANOVA). Expression of CD31 fell significantly from baseline (median fluorescence intensity, (MFI) 7363.5 ± 1492.2) to 4h (4960 ± 1298.3), 6 h (4769 ± 1286.3) and 24h (2680.7 ± 713.9 , $p = 0.002$, ANOVA; **Figure 5**).

Extended phenotypic analysis (CD34, CD309, CD144 and CD146) showed stable marker expression from harvest to 2 h. There was a non-significant trend towards reduced CD144 expression at 24h, all other markers remained stable ($p > 0.05$ for all comparisons, ANOVA with Tukey multiple comparison's; **Figure 6**).

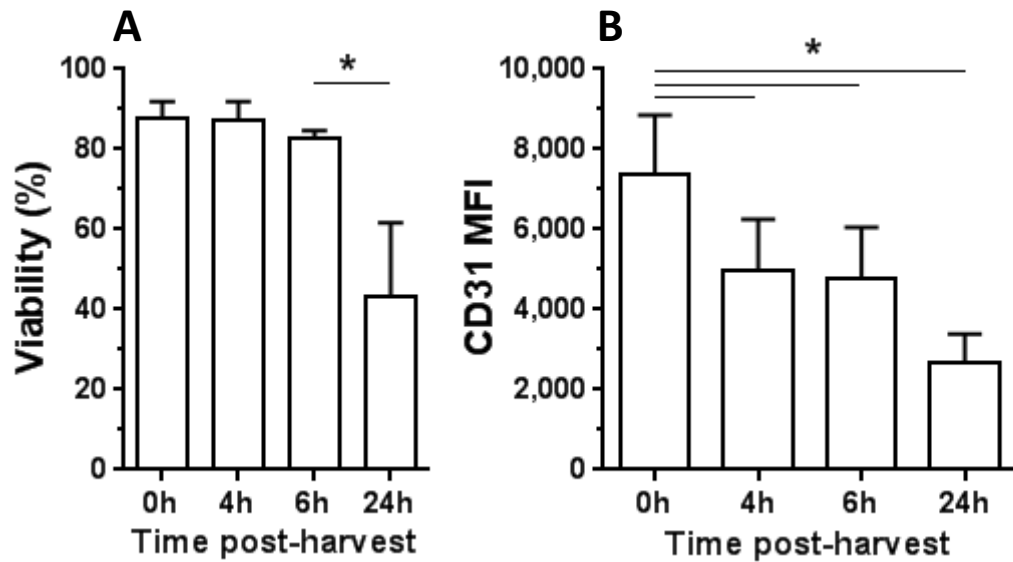


Figure 5: Functional stability of process validation cultures.

Viability of EOCs remained high for up to 6h after harvest but fell significantly by 24h (43 ± 18.6 $p=0.02$, ANOVA). Expression of CD31 fell significantly over time from baseline at 0 h to 24h ($p=0.002$, ANOVA), though there was stabilisation of expression between 4-6h ($*=p<0.05$, ANOVA with Tukey multiple comparisons; $N=4$).

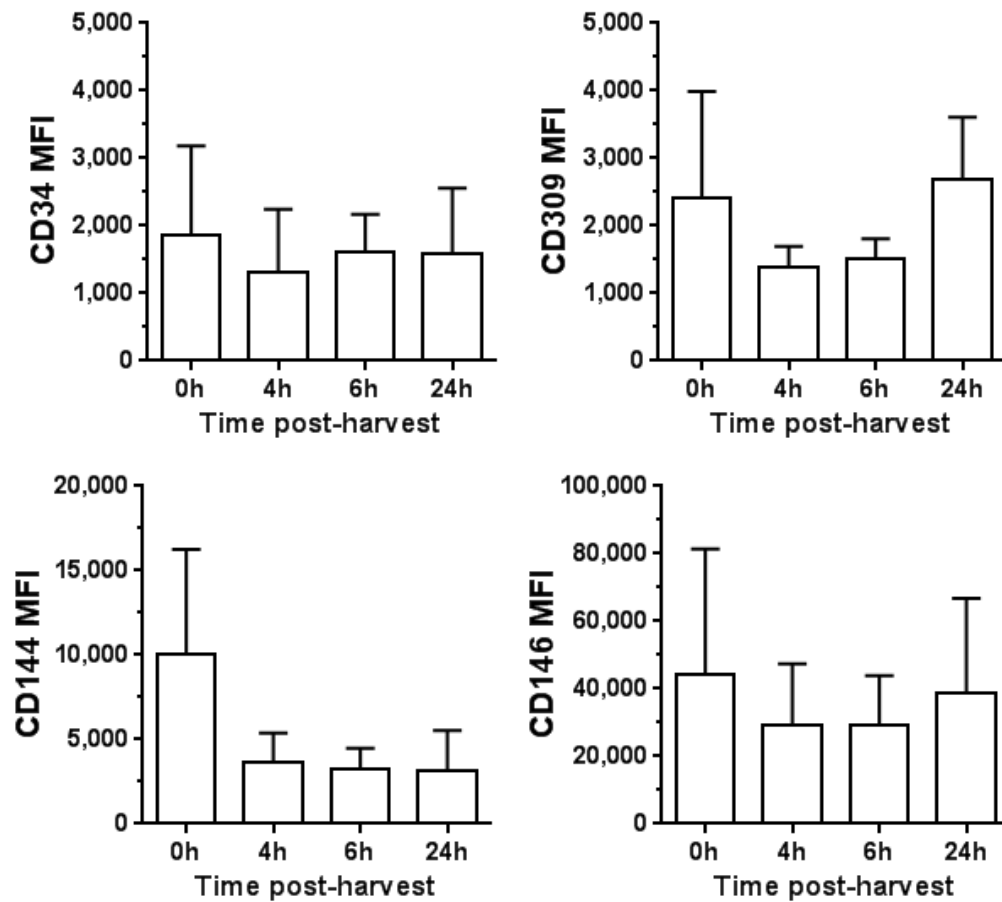


Figure 6: Extended phenotypic analysis of process validation cultures with stability assessment.

Extended panel analysis of the EOC demonstrate relative stability of functional marker expression over time. There were no significant differences detected in each marker, though there was a trend to reduced expression in CD144 by 24h post-harvest (*= $p>0.05$, ANOVA with Tukey multiple comparisons). N=4.

6.4 DISCUSSION

We describe the successful translation of a preclinical protocol for the isolation and expansion of late-outgrowth endothelial cell culture through to a GMP-compliant protocol. Using this protocol, we have demonstrated reliable culture of phenotypically stable EOCs with high viability. Cell viability and phenotype remain stable for up to 6h.

Whilst our research protocol was well-established and effective, it contained multiple components of animal origin posing a problem for GMP compliance which aims to fully define each product used in manufacture and exclude if possible any component of human or animal origin. Our original protocol employed rat's tail collagen to coat cultureware and facilitate cell attachment, our medium was also supplemented with foetal calf serum and contained research-grade heparin, all of which are potentially problematic for the purposes of GMP compliance.

For cell attachment we trialled uncoated cultureware which was incapable of supporting EOC culture, presumably because EOCs needs some form of cell/ECM interaction to thrive. We then trialled Cellstart™. Product literature suggested that Cellstart™ would support human embryonic and mesenchymal stem cells although there were no reports of EOC isolation. EOC proliferation was not supported by Cellstart™ but was by another commercially-available synthetic ECM-mimetic (Purecoat™). Cellstart, like many ECM-mimetic

substances, is composed largely of fibronectin (Hughes *et al.* 2011), whilst Purecoat™ is composed largely of synthetic Collagen I which is known to be important to EOC isolation (Yoder 2009) and this may explain our differential experience with these substitutes.

Media substitution was more straightforward and involved switching from research-grade to a GMP-compliant version of the same endothelial media from the same supplier. The basal media is provided with a kit of growth factor supplements which are added when the medium is made-up immediately prior to use. Each of these growth factor supplements were risk assessed and during this process one component- insulin provided with the media which was switched for clinical grade insulin. Nutrient supplementation of medium is required to support EOC proliferation and it is therefore not surprising that unsupplemented media was unable to support EOC proliferation. Only AB donor serum and not autologous serum, human albumin solution, nor Xerum-free™ (a commercially-available serum-free media supplement) was able to support EOC proliferation. The mechanism behind the failure of human albumin solution, autologous serum and Xerum-free™ in supporting OEC proliferation are unclear. It is possible that there is a factor in AB donor serum which is absent in human albumin solution and is required for cell proliferation, it is also possible that a substance was present in the autologous serum which inhibited cell proliferation (blood group of participants was not recorded).

Colonies were isolated from all four donors in the process validation phase of our study. These cells were phenotypically identical to research-grade EOCs (Yoder *et al.* 2007; Tura *et al.* 2013) with typical endothelial cobble-stone morphology, high expression of the endothelial cell surface marker CD31 and low retention of CD45+ contaminating cells from the initial mononuclear cell culture.

Sequential analysis post-harvest demonstrated that whilst there is a steady decline in CD31 and CD144 expression, other endothelial markers (CD34, CD309) remain stable, even at 24h. Viability of the cell product was stable out to 6h but fell at 24h. Whilst not a substitute for functional assays, the stability of cell surface markers and cell viability afforded reassurance that the EOC product, administered on the day of harvest will represent a viable and functional cell population

Late EOC colonies were isolated from all donors, the final cell yield varied between donors and was somewhat lower in the four process validation runs than in research grade cultures. This likely reflects biological variation between donors, although the reasons for this difference were not examined in detail in this protocol. Our results are in keeping with published work by our group (Mitchell *et al.* 2017) in which we studied an identical patient cohort and were able to isolate EOCs successfully from in 30 of 48 patients.

Other groups report similar variability in EOC culture success. Increased efficacy of EOC isolation has been reported from patients with inflammatory conditions (De Villeroché *et al.* 2010), burns (Rignault-Clerc *et al.* 2013) and acute myocardial infarction (Massa *et al.* 2009). However, all donors in our study were drawn from a stable outpatient population, albeit with cardiovascular risk factors highly represented. Our findings are consistent with recent work in a stable outpatient population (Dauwe *et al.* 2016) in which colony isolation was positively correlated with increasing age but no other predictors of colony yield were identified.

Previous studies examining myocardial and vascular regeneration in man have often used heterogeneous cellular populations and this may underlie the modest improvement in cardiac function seen to date (Fisher *et al.* 2017). Given that angiogenesis or vasculogenesis and the paracrine effects of these cells are likely to be crucial to tissue regeneration (Sieveking *et al.* 2008), it is possible that a more differentiated, endothelial-specific cell population will yield better results.

To date EOC are the only candidate cellular product for vascular regeneration shown to incorporate into recipient vessels, albeit in a mouse model of angiogenesis (Padfield *et al.* 2014; Sieveking *et al.* 2008; Dubois *et al.* 2010; Tura *et al.* 2013; Barclay *et al.* 2012) and increase vessel density both in a swine model of myocardial infarction (Dubois *et al.* 2010) and in murine models of hind-limb ischaemia (Dauwe *et al.* 2016; Minami *et al.* 2015; Sieveking *et al.*

2008). We therefore believe that EOCs represent a novel and promising agent for trials of vascular regeneration.

We have defined a GMP-compliant culture protocol which will allow human administration and examination of the behaviour of EOCs *in vivo* in man. Using this protocol we are able to reliably isolate EOC and have comprehensively characterised the resultant cells demonstrating them to be EOCs. This will allow us to proceed confidently to trials of human administration, initially carrying out small scale cell tracking studies. If these are promising we would anticipate seeking regulatory approval for *in vivo* in man trials of these cells as agents for vascular regeneration.

Chapter 7

Labelling late-outgrowth endothelial cells with ^{18}F -Fluorodeoxyglucose

SUMMARY

Endothelial outgrowth cells (EOCs) can be isolated from the peripheral blood and have been shown to contribute to vascular repair in animal models, they are therefore a potential agent for regenerative medicine in chronic ischaemic conditions. As a prelude to a cell tracking study, we sought to characterise the feasibility of labelling using ^{18}F -FDG and the effect of the radiotracer on labelled cells.

Human EOCs were incubated with a range of ^{18}F -FDG concentrations and cellular tracer incorporation, acute viability and cellular proliferation were assessed. From the resulting dose response curves an incubating concentration of 25 MBq/mL was chosen for scaled-up experiments with cellular incorporation, tracer leak and cellular viability, adhesion and cell cycle analysed.

Labelling with ^{18}F -FDG had no effect on acute cell viability but there was a strong negative correlation between incubating ^{18}F -FDG concentration and cellular proliferation (Pearson $r=-0.9$, $p<0.001$). Labelling 25×10^6 cells using a mean initial incubating activity of 250 MBq ^{18}F -FDG, the mean activity in the cell pellet was 6.04 ± 1.15 MBq giving a mean labelling efficiency of 2.4%. When compared to control cells, no difference was detected in viability, adhesion or cell cycle analysis.

EOCs can be effectively labelled with ^{18}F -FDG and whilst cellular proliferation is significantly reduced, there is no effect on acute viability or cellular function. Alongside the development of a GMP-compliant protocol for EOC culture, this paves the way for a tracking study of EOCs in man.

7.1 INTRODUCTION

With proven ability to incorporate into host vessels and stimulate vasculogenesis when administered in animal models (Barclay *et al.* 2012; Dubois *et al.* 2010), late outgrowth endothelial cells are a promising therapy for ischaemic vascular conditions.

A key step in the development of any targeted cell therapy is to demonstrate adequate delivery to and retention of the cell product in the target tissue, organ or site of injury. Without this confirmation, it is difficult to provide ethical, financial and practical justification for the subsequent endeavour required to undertake large clinical trials. As well as demonstrating bioplausability prior to moving to more expensive and involved studies of efficacy, these preliminary studies allow the optimisation of the cell population and delivery method employed. Whilst incorporation of EOCs into vessel walls in animal models of ischaemia has been observed, (Barclay *et al.* 2012), homing *per se* has yet to be demonstrated.

Acute myocardial infarction and chronic ischaemic cardiomyopathy are the cardiovascular conditions most frequently examined in tracking studies. In keeping with therapeutic trials, tracking studies have focused predominantly on undifferentiated cell populations, such as bone marrow or peripheral blood CD34+ cells. These studies have largely used intracoronary administration and

have demonstrated myocardial retention of infused cells with delayed imaging time points between 1 and 48h (**Table 1**).

| Condition | n | Cell type | Isotope | Delivery route | Percentage of cells retained in myocardium | Imaging time point |
|---------------------------------|----|------------------------------|---------------------|----------------|--|--------------------|
| Acute MI | | | | | | |
| Kang (2006) | 14 | Haematopoietic stem cells | ¹⁸ F-FDG | IC | 1.5 | 20h |
| Hofmann (2005) | 6 | Bone marrow cells | ¹⁸ F-FDG | IC | 2.9 | 1h |
| Hofmann (2005) | 3 | Bone marrow (CD 34-enriched) | ¹⁸ F-FDG | IC | 25.7 | 1h |
| Blocklet (2006) | 6 | Peripheral blood 34+ cells | ¹⁸ F-FDG | IC | 5.5 | 1h |
| Kurpisz (2007) | 8 | Peripheral blood progenitor | ¹¹¹ In | IC | 9.4 | 24h |
| Penicka (2007) | 3 | cells | ¹¹¹ In | IC | 6.2 | 2h |
| Mesquita (2005) | 5 | Bone marrow (CD 34-enriched) | ⁹⁹ Tc | IC | 2.9 | 24h |
| Ischaemic cardiomyopathy | | | | | | |
| Dedobbeleer (2009) | 7 | Peripheral blood CD34+ | ¹⁸ F-FDG | IC | 3.4 | 1h |
| Hofmann (2005) | 6 | Haematopoietic stem cells | ¹⁸ F-FDG | IC | 1.6 | 1h |
| Caveliers (2007) | 3 | Peripheral blood CD 133+ | ¹¹¹ In | IC | 7.5 | 24h |
| Kurpisz (2007) | 11 | Peripheral progenitor cells | ¹¹¹ In | IC | 4.8 | 24h |
| Mesquita (2005) | 5 | Bone marrow cells | ⁹⁹ Tc | IC | 1.6 | 24h |

The main imaging modalities for human cell tracking studies are SPECT and PET which require labelling of cells with a radioisotope (**Table 1**). SPECT and PET are similar in that they use gamma cameras to triangulate a radioactive source with the main difference being the difference in the radioisotopes used- SPECT uses Gamma emitters which generally have a longer half-life whereas PET uses positron-emitting isotopes. The major considerations for these labelling agents relate to efficiency of labelling (i.e. incorporation of sufficient activity to allow visualisation in the scanner) and the effect of the labelling process on cellular function, with the ideal agent being avidly taken up by cells whilst having no deleterious effects. Labelling efficiency reported in studies to date is highly variable, even across studies using the same isotope (McColgan *et al.* 2011). Some of this variation may reflect insufficient washing of labelled cells following isotope incubation, resulting in an erroneously high reported labelling efficiency (Hofmann *et al.* 2005).

^{18}F -Fluorodeoxyglucose is the most commonly used PET tracer (Chan *et al.* 2012) and is discussed below. Alternative PET tracers have been used for pre-clinical cell labelling work, notably ^{18}F -fluorothymidine (^{18}F -FLT) and Hexadecyl-4- ^{18}F - fluorobenzoate (^{18}F -HFB). ^{18}F -FLT is a nucleotide analogue and accumulates in actively dividing cells, it has been used clinically in cancer imaging (Szysko *et al.* 2016) and is effective in pre-clinical trials of cell labelling (MacAskill *et al.* 2017). ^{18}F -HFB is a lipophilic molecule which is taken up by the cell membrane and whilst effective cell labelling has been demonstrated in pre-

clinical work, there are concerns over acute toxic effects on cells (Zhang *et al.* 2012) and its use remains restricted to pre-clinical work.

¹⁸F-Fluorodeoxyglucose is a glucose analogue with a hydroxyl group substituted for the radioactive isotope fluorine and is the most widely used radioisotope in cell tracking studies (Rosado-de-Castro *et al.* 2014). The tracer is trapped intra-cellularly following phosphorylation, accumulating in cells in proportion to glycolytic activity and thus accumulates in metabolically active tissues. This underlies its utility in cancer imaging where the free tracer preferentially localises to the more metabolically active tumour cells.

Following intra-venous administration, 20% of ¹⁸F-FDG is eliminated renally with the remainder taken up by the tissues by insulin dependent and independent transport (Swanson *et al.* 1990) with a half-life of 110 minutes. This relatively short half-life of ¹⁸F-FDG and the significant loss of the tracer over the first two hours post-labelling (Doyle *et al.* 2007; Zhang *et al.* 2012) limit its use in long-term tracking of cell fate. However, ¹⁸F-FDG remains useful for short-term cell tracking studies, especially in vascular imaging where the superior spatial resolution of PET-CT over SPECT (Chan *et al.* 2012) is an important consideration and has been employed extensively in cardiovascular cell tracking studies (Doyle 2007; Hofmann *et al.* 2005; Dubois *et al.* 2010; Blocklet *et al.* 2006).

The deleterious effects of radiotracers on cellular function is well documented (Berge *et al.* 1983; Faivre *et al.* 2016) and limits the dose of tracer that can be used. The majority of cell- tracking studies report reassuring post-labelling viability in the region of 90%. However, cell death is one extreme of cellular dysfunction and thus milder degrees of cellular damage resulting in functional impairment may be under-reported. To address this, some investigators have undertaken more detailed functional analysis of labelled cells with some reporting retained proliferative and differentiation potential and no DNA damage (Lang *et al.* 2013; Elhami *et al.* 2011) whilst others suggest that cellular migration and proliferation is impaired (Faivre *et al.* 2016).

We sought to demonstrate whether EOCs could be effectively labelled with ^{18}F -FDG and to define the effect of the labelling process on cellular viability and function.

7.2 METHODS

7.2.1 ^{18}F -FDG incorporation

Confluent flasks of EOCs were trypsinised, cells were then counted and resuspended in saline supplemented with 0.5% human albumin solution (Lonza, Switzerland). Cells were then incubated with ^{18}F -FDG (concentration of 5-50MBq/ml) for 30 minutes at 37°C and agitated at 300 Hz using an automated mixer (Thermomixer®, Eppendorf, Germany). Unincorporated tracer was removed with a three-step washing protocol with saline and 300 *g*, 5-minute

centrifuge spins. Samples of the stock ^{18}F -FDG solution, washing supernatant (for unincorporated extracellular activity at each wash) and the final resuspended cellular pellet were read in a gamma counter (1470 Wizard, PerkinElmer lifesciences, USA). All counts were decay-corrected to the time of incubation and expressed in MegaBecquerels.

Published data (Eriksson et al. 2009; Paik et al. 2002; Doyle et al. 2007) and our own preliminary experimental work (data not shown) suggested that incorporation is optimal when tracer and cells are incubated at 37°C in low-glucose medium with the addition of insulin (10 units/mL, Humulin S, Eli Lilly, UK) and these conditions were used for all experiments.

7.2.2 Assessment of cell viability following ^{18}F -FDG exposure

Cells (5×10^5) were washed in PBS, they were then incubated with Zombie GreenTM dye (Biolegend, USA) (1:1000 solution 20 minutes). Samples were fixed and analysed using a 4-laser flow cytometer (LSR Fortessa BD biosciences, USA). Analysis was performed offline using proprietary software (Flowjo, treestar, USA). Gating strategies are included as an appendix.

7.2.3 Assessment of proliferation following ^{18}F -FDG exposure

Following labelling and washing, cells were re-suspended in endothelial growth medium (EBM-2, Lonza, Switzerland) and counted. 1×10^5 cells were plated to rat's tail collagen-coated 6-well plates (BD Biosciences, USA) and endothelial

growth medium (EBM-2, Lonza, Switzerland) was added. Non-adherent cells were removed at 24h and medium was thereafter changed twice per week. Cells were counted at 24h, 3 and 7 days.

Dose-response curves were constructed, plotting ^{18}F -FDG incorporation and cellular viability and proliferation against the initial incubating concentration of ^{18}F -FDG . From these curves an ^{18}F -FDG concentration of 25 MBq/mL was chosen and cell numbers were then scaled up to those anticipated in a trial of human administration for subsequent experiments. In subsequent characterisation of labelled cells (viability, leakage and cell cycle analysis), labelled cells were compared to controls. Volumes and cell numbers are stated in all figures and the sequence of experimental work is outlined in **Figure 1**.

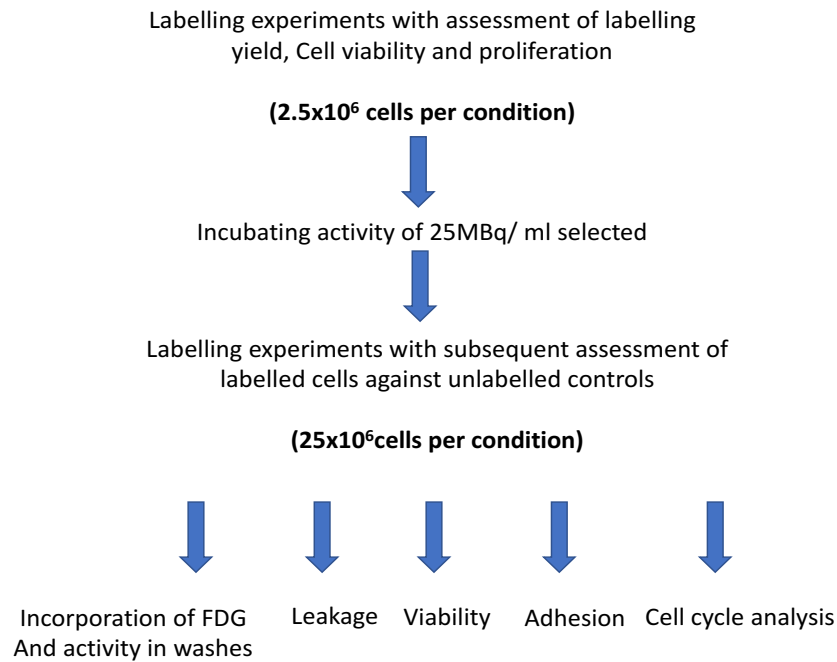


Figure 1. Sequence of experimental work with cell numbers for labelling experiments

7.2.4 Assessment of ¹⁸F-FDG leakage following labelling

Labelled cells were suspended in endothelial basal media at 37°C. After one hour, the suspension was centrifuged and the activity of the supernatant measured. The cell pellet was re-suspended and incubated at 37°C for a further hour before centrifugation and measurement of the supernatant performed to determine the extent of ¹⁸F-FDG leakage from cells over a two-hour period.

7.2.5. Assessment of EOC adhesion following ^{18}F -FDG exposure

EOCs were washed and suspended in EBM-2 (Lonza, Switzerland). Cells (4×10^4) were added to 6-well plates coated with Rat's tail collagen with 1 mL of EBM-2 (Lonza, Switzerland) and incubated at 37°C for 30 minutes. Plates were then washed three times and cells were fixed using formalin. Imaging was carried out using inverted light-source microscopy (Zeiss Observer, Carl Zeiss, Germany) and adherent cells expressed as percentage of the original cell number.

7.2.6. Assessment of cell cycle following ^{18}F -FDG exposure

Collagen-coated T25 flasks (Corning, USA) were plated with 1×10^6 cells and cultured as above for cell cycle analysis. At 48 hours, cells were trypsinised (TrypLE™, Thermo Fisher, USA), DAPI prep™ (Sony Biotech, USA) was added and 20,000 events were acquired on a 4-laser flow cytometer (LSR Fortessa, BD Biosciences, USA). Analysis was performed offline using proprietary software (Flowjo, treestar, USA).

7.3 RESULTS

7.3.1 ^{18}F -FDG Incorporation dose-response, acute toxicity and effect on cellular proliferation

^{18}F -FDG incorporation increased up to a plateau at an incubating concentration of 25 MBq/mL (**Figures 2 and 3**). There was no effect of tracer incorporation on acute cell viability (**Figure 2**) but EOC proliferation at seven days was strongly negatively correlated with ^{18}F -FDG incorporation ($r=-0.9$, $p<0.001$; **Figure 3**).

An initial incubating activity of 25 MBq/mL was chosen for scaled-up experiments during which further analysis of the fidelity of the labelling process and functional assessment of the labelled cells was carried out. The results below relate to experiments using 25×10^6 cells incubated in a volume of 10 mL with 250MBq (25MBq/mL) of ^{18}F -FDG and 100 units of insulin (Humulin S, Lily, USA).

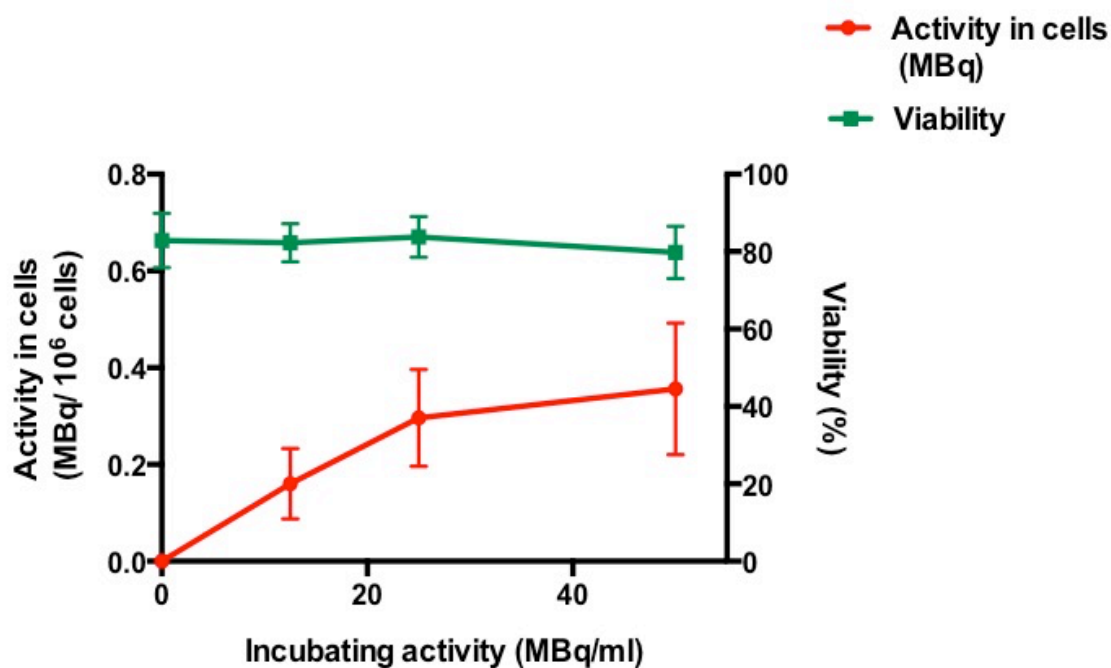


Figure 2: ^{18}F -FDG incorporation and acute toxicity. Cellular ^{18}F -FDG incorporation started to plateau at an initial incubating concentration of 25MBq/ ml. There was no obvious acute toxic effect of ^{18}F -FDG as incubating concentration and incorporated activity increased. Experiments used 2.5×10^6 cells per tube, each data point represents 3 biological repeats with 2 technical repeats performed per experiment.

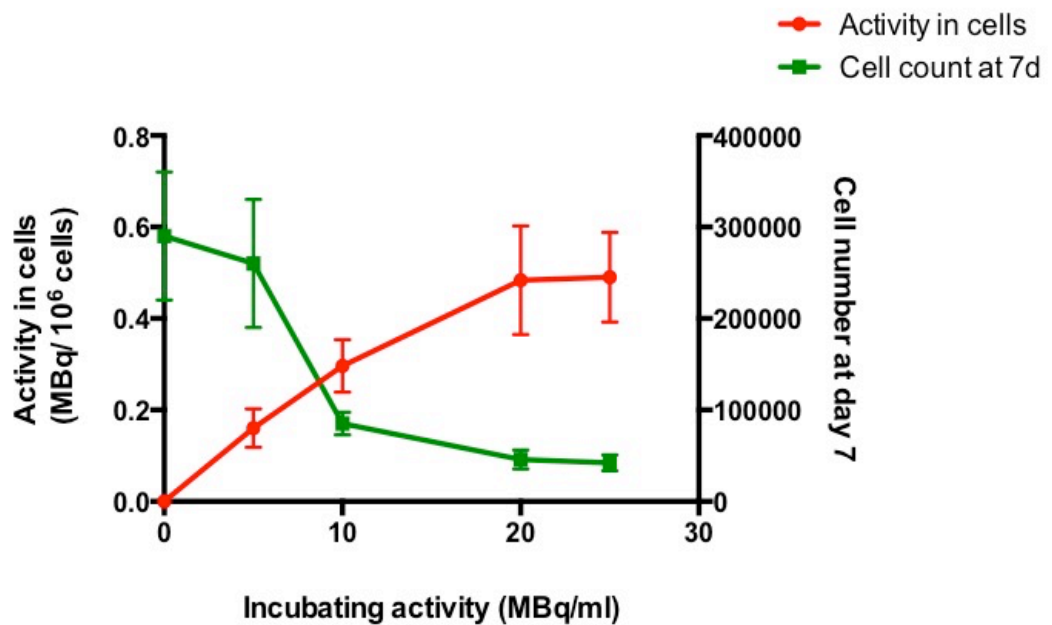


Figure 3: ^{18}F -FDG incorporation and cellular proliferation. Cellular ^{18}F -FDG uptake increased to a plateau at an initial incubating activity of 20-25 MBq/mL. There was a strong inverse relationship between incorporated activity and cellular proliferation as measured by cell number following 7 days of culture. Experiments used 2.5×10^6 cells per tube, each data point represents at least 3 biological repeats with 2 technical repeats performed per experiment.

7.3.2. ¹⁸F-FDG incorporation and washing efficacy

Using a mean initial incubating activity of 250 MBq (25MBq/mL) ¹⁸F-FDG, the mean activity in the cell pellet was 6.04±1.15 MBq giving a mean labelling efficiency of 2.4%. As expected there was a sequential reduction in extra-cellular activity on serial washes. The extra-cellular activity after 2 washes was 2.1±0.73 MBq (**Figure 4**).

7.3.3. ¹⁸F-FDG Leakage following labelling

There was significant leak of ¹⁸F-FDG from cells with 19.65±3.2% and 33.05±5.2% lost by 1 and 2 hours respectively (**Figure 5**).

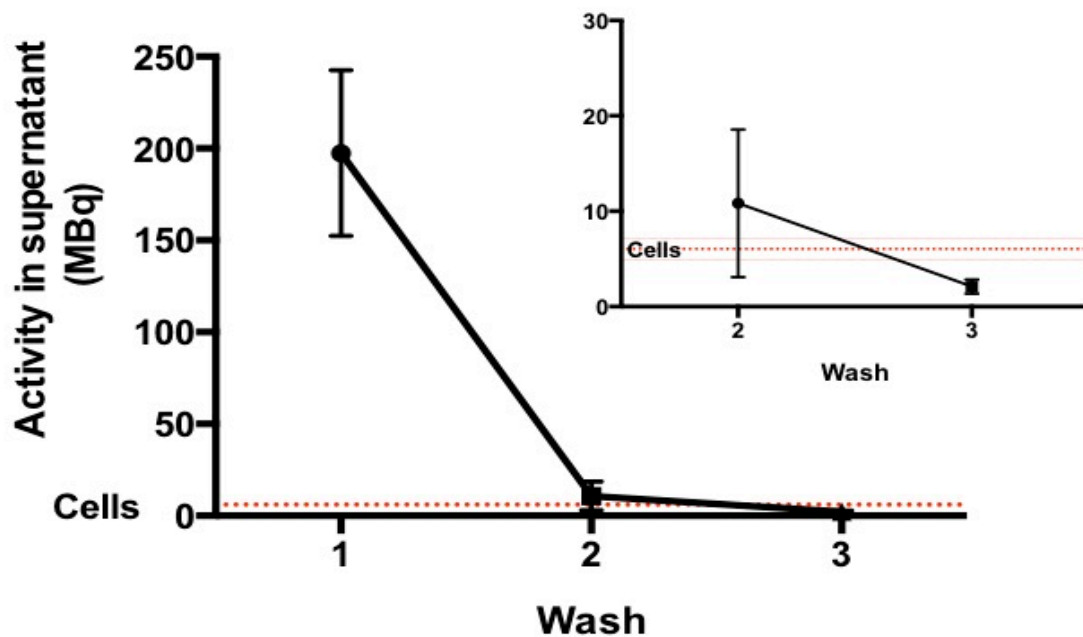


Figure 4: Activity in washing supernatant and cellular pellet. The black line represents the extra-cellular activity at each wash and the red dotted line represents the intra-cellular activity after three washes. There was marked contrast between the activity in the final wash and the intra-cellular activity (2.1 ± 0.73 versus 6.04 ± 1.15 MBq respectively) **see inset**. $n=4$ with 2 technical repeats. Mean cell number $19.33 \pm 6.2 \times 10^6$, incubation in 10 mL saline with 10% AB serum and 100 U of insulin

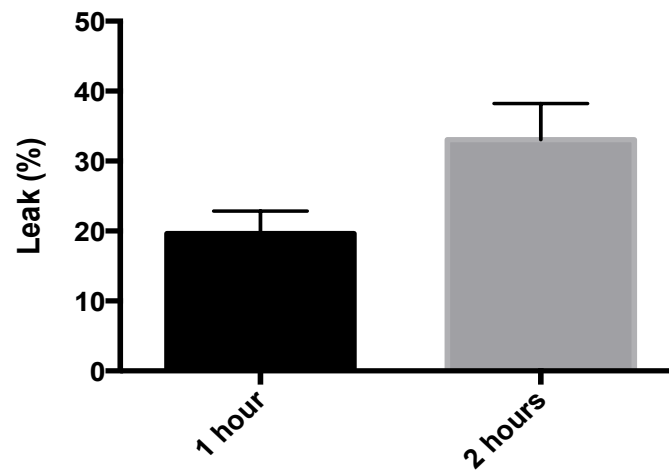


Figure 5: Cellular ^{18}F -FDG leakage following labelling. $19.65 \pm 3.2\%$ and $33.05 \pm 5.2\%$ of activity was lost from cells by 1 and 2 hours respectively.

7.3.4. EOC viability following labelling compared to controls

There was no significant difference in acute viability between cells exposed to 25MBq/mL ^{18}F -FDG and controls ($87.33 \pm 6.77\%$ *versus* $92.67 \pm 0.67\%$ respectively $p=0.48$; **Figure 6**).

7.3.5. EOC adhesion following labelling compared to controls

Adhesion was no different between cells exposed to 25MBq/mL ^{18}F -FDG and controls (22.25 ± 4.7 *versus* $20.3 \pm 2.8\%$ respectively $p=0.2$; **Figure 6**).

7.3.2.5 Cell cycle analysis

There were no differences between cells exposed to 25MBq/mL ^{18}F -FDG and controls in terms of the percentage of cells in the G_0/G_1 (85 ± 6.2 *versus* $88.1 \pm 8.6\%$ for control and ^{18}F -FDG respectively, $p=0.75$), S (8.6 ± 4 *versus* $2.8 \pm 1.9\%$, $p=0.09$) or G_2M (5.2 ± 2.17 *versus* $7.7 \pm 5.44\%$ $p=0.5$) phases of the cell cycle (**Figure 6**).

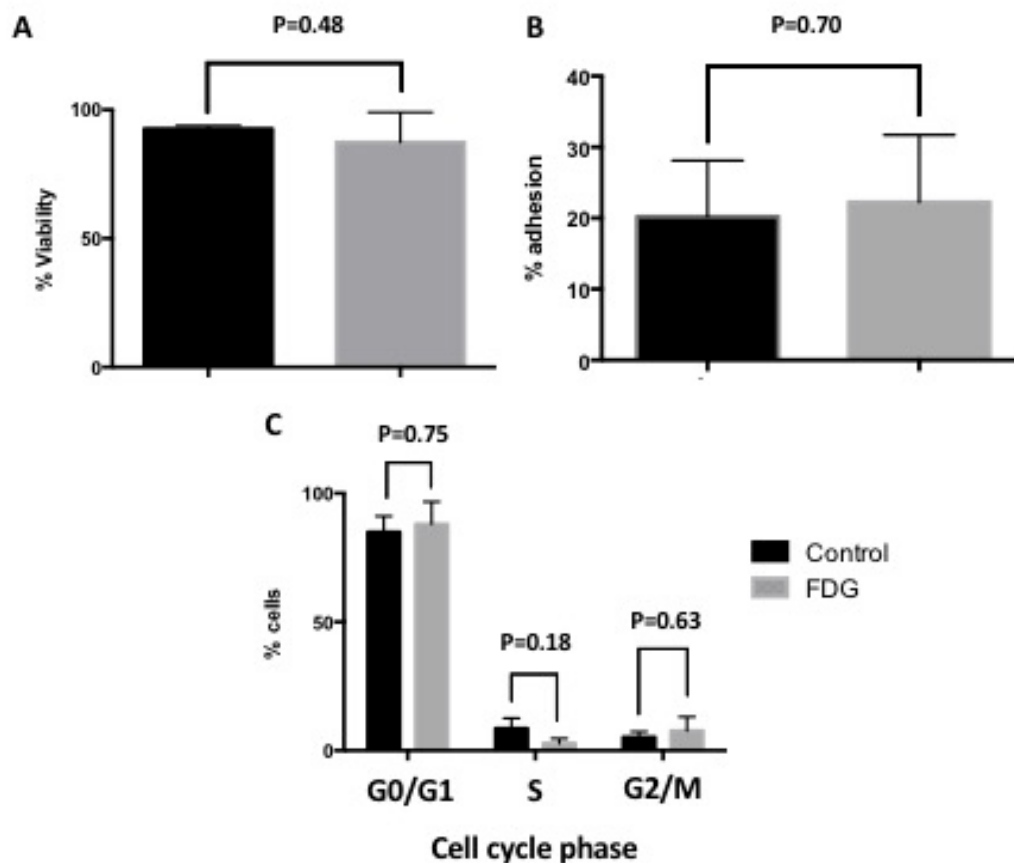


Figure 6: Functional analysis of labelled cells. There was no difference in acute viability between labelled and control cells (87.33 ± 6.77 versus $92.67 \pm 0.67\%$ viability respectively $p=0.48$) **A**. Cellular adhesion also remained unchanged between labelled and control cells (22.3 ± 4.7 versus $20.3 \pm 2.8\%$ adhesion respectively, $p=0.7$) **B**. There was no difference between labelled and control cells on cell cycle analysis **C**. For all experiments 25×10^6 cells were incubated in 10 mL of saline supplemented with 10% AB serum with an ^{18}F -FDG activity of 25 MBq/mL and 100 units of insulin. Each data point represents 3 biological repeats with 2 technical repeats.

7.4 DISCUSSION

This series of in vitro studies demonstrate that labelling EOCs with ^{18}F -FDG is feasible. Although there was evidence of attenuation of cell proliferation over seven days, labelling with ^{18}F -FDG had no acute effect on EOC viability, cell cycle, or the ability of cells to adhere to collagen in vitro.

Although EOCs were successfully labelled with ^{18}F -FDG, the efficiency was low with 2.4% of the incubating activity incorporated into cells in the final protocol. Reports of labelling efficiency in previous studies are highly variable with labelling efficiencies of 4% to 99% reported (Blocklet *et al.* 2006; Hofmann *et al.* 2005; Kang *et al.* 2006; Eriksson *et al.* 2009). Differences in the number of washing steps and centrifugation protocols (McColgan *et al.* 2011) may result in inadequate removal of unbound tracer may account for some of the observed variation.

Administration of a high concentration of unincorporated ^{18}F -FDG may explain the low rates of cellular retention at the site of injection reported in some human trials (Hofmann *et al.* 2005) as free tracer is subject to mechanisms of distribution metabolism and elimination in way that intra-cellular tracer is not. Examination of the washings in the current series of studies provide reassurance that the vast majority of ^{18}F -FDG in our final labelled product is intra-cellular and not free. Our study suggests that, for any given labelled cell product, >75% of the activity administered will be intra-cellular.

Preclinical studies in a variety of cell types demonstrate that ^{18}F -FDG labelling may cause DNA damage (Elhami *et al.* 2011; Minana *et al.* 2015; Kashino *et al.* 2014) and impaired cellular proliferation (Minana *et al.* 2015; Elhami *et al.* 2011; Wolfs *et al.* 2013). In our studies, we observed attenuation of EOC proliferation at all doses of ^{18}F -FDG. Interestingly the G₂M accumulation (a checkpoint in the cell cycle prior to mitosis with an increased proportion of cells in this phase suggestive of DNA damage) previously reported following gamma radiation exposure (Fujii *et al.* 1999) was not observed. Despite attenuation of proliferation, EOC viability and function (as defined by adhesion assay) remained intact acutely, in keeping with previous work which has demonstrated retained functionality following labelling despite impaired cellular proliferation (Lang *et al.* 2013; Elhami *et al.* 2011).

Whilst the fidelity of labelling at the point of injection is clear, leakage is an issue with 19% of incorporated tracer escaping by 1h and 33% by 2h. Traditional wisdom holds that following cellular uptake, ^{18}F -FDG is phosphorylated by hexokinase with the resultant ^{18}F -FDG-phosphate unable to either diffuse out of the cell or to undergo glycolysis (due to the lack of a hydroxyl group normally present on glucose but in the case of ^{18}F -FDG substituted for fluoride) and therefore accumulates intra-cellularly. However, significant leak has been reported for various cell types (Doyle *et al.* 2007; Lang *et al.* 2013) and is likely due to either saturation of hexokinase (which phosphorylates glucose) activity, intra-cellular dephosphorylases or cell death and release of cytoplasmic

contents. The major consequence of ^{18}F -FDG leakage from cells is a potential reduction in the cellular signal rather than non-specific uptake of free tracer by body tissues given that the absolute amount of activity injected is negligible when distributed to total body water.

In conclusion, labelling of late outgrowth cells with ^{18}F -FDG is feasible and whilst longer term proliferation of labelled cells is significantly impaired, acute cellular function and viability are not. We believe these studies provide support for the use of ^{18}F -FDG as a label for EOC and alongside a GMP-compliant protocol for EOC culture, this will allow the tracking of these cells when administered *in vivo* in man.

Chapter 8

Conclusions and future directions

8.1 BACKGROUND

Endothelial injury is the crucial initiating event in atherosclerotic disease and is also ubiquitous following percutaneous vascular intervention. As the cellular mechanisms of repair largely determine vessel outcome, improving our understanding of this response will facilitate the development of novel treatments for chronic ischaemic conditions and optimisation of percutaneous interventions.

The discovery over twenty years ago of a circulating endothelial progenitor cell capable of incorporating into damaged vessels (Asahara *et al.* 1997) led to great interest in endothelial progenitor cells as a potential target for pharmacological manipulation and substrate for cellular therapy. Whilst some early work seemed to confirm the utility of EPC numbers as biomarkers for vascular health (Werner *et al.* 2005), subsequent studies have failed to confirm this (Padfield *et al.* 2013; Padfield *et al.* 2014; Thomas *et al.* 2008) and attempts to manipulate EPC populations using stent technology have as yet been unsuccessful (Klomp *et al.* 2011).

The description of late-outgrowth endothelial cells (Yoder *et al.* 2007), a more highly differentiated cellular population thought to originate from a circulating endothelial progenitor cell, represented a new avenue for regenerative and translational research. These cells resemble mature endothelium, are highly

proliferative (Timmermans *et al.* 2007) and incorporate into damaged host vessels in animal models (Barclay *et al.* 2012). They are therefore a potential agent for vascular regeneration and the work presented in this thesis sought to define their role in a mechanical vascular injury model and lay the foundations for human administration in a cell tracking study.

The radial artery is the predominant route of vascular access for coronary angiography in the United Kingdom ('BCIS National Audit of Percutaneous Coronary Interventions' 2014) and reduces bleeding and vascular complications when compared to femoral access (Cruden *et al.* 2007; Jolly *et al.* 2011). Despite the obvious advantages of this approach, subclinical arterial injury at the time of catheterisation (Yonetsu *et al.* 2010; Di Vito *et al.* 2013) leading to impaired vasomotor function (Dawson *et al.* 2010; Madssen *et al.* 2006; Burstein *et al.* 2007) has been described. This, combined with the accessibility of the human forearm led us to believe that the radial artery may be a valuable model of human arterial injury *in vivo*.

Using flow-mediated dilatation and optical coherence tomography, we characterised the radial artery as a model of vascular injury, defining the structural and functional consequences of transradial cardiac catheterisation, the accompanying cellular profiles and the modifying effects of these profiles on vessel recovery.

Subsequently, with a view to conducting a cell-tracking study in man our research grade protocol for EOC culture was then translated for GMP compliance and a protocol for labelling these cells with the PET tracer ^{18}F -Fluorodeoxyglucose was developed.

8.2 SUMMARY OF FINDINGS

8.2.1 Transradial catheterisation is associated with a low incidence of OCT-detectable injury, ubiquitous endothelial denudation and reversible impairment of arterial function

The radial artery following transradial cardiac catheterisation is an accessible and promising model of vascular injury which we ultimately hope to utilise to study the behaviour of EOC in man. Whilst both structural injury (Yonetsu *et al.* 2010; Di Vito *et al.* 2013) and functional impairment (Dawson *et al.* 2010; Madssen *et al.* 2006; Burstein *et al.* 2007) have been described separately, no study has comprehensively documented the structural changes, functional impairment and recovery and the accompanying cellular profiles. We sought to address this and in so doing fully characterise our model which we plan to use in future work.

Using OCT, intra-vascular imaging of radial arteries was carried out upon withdrawal of the vascular sheath. Significant injury was detected on OCT imaging in 24% of patients which was surprisingly low in the context of previous studies (Yonetsu *et al.* 2010; Di Vito *et al.* 2013). This is likely to reflect the low incidence of patients undergoing repeat procedures, and the larger baseline radial artery diameter in our cohort. In addition to imaging, we examined the explanted vascular sheaths in a subset of our patients, isolating cells by centrifugation and identifying endothelial cells by flow cytometry. In

doing so we demonstrated that large numbers of endothelial cells were removed by the vascular sheath in all cases.

Longitudinal functional assessment of the radial artery was carried out using flow and nitrate-mediated dilatation. This demonstrated a reversible impairment of endothelium dependent and independent function which peaked at 24 hours and recovered by 3 months and 1 month respectively. This likely reflects the endothelial denudation demonstrated by our examination of angiography sheaths with recovery representing reconstitution. Better understanding of the conditions influencing this process of recovery may lead to novel cardiovascular therapies and could be achieved by using the radial artery as a model of injury and repair.

8.2.2 Neither circulating EPC numbers nor EOC function influence vessel recovery following injury

Peripheral blood EPC numbers have previously been shown to be negatively correlated with cardiovascular events (Werner *et al.* 2005) although more recent studies are less clear (Padfield *et al.* 2013). Whilst studies have documented EPC profiles following percutaneous coronary intervention (Thomas *et al.* 2008; Padfield *et al.* 2014), cellular predictors of vessel recovery have not been examined.

EPC profiles and EOC function were measured at baseline and 24 h post-angiography and the influence of these cells on functional recovery of the radial artery were examined. Neither peripheral blood EPC concentration nor the function of isolated EOCs predicted recovery of vascular function following injury. CD34⁺ cell concentration was negatively correlated with both baseline endothelial function and recovery following injury. EOC function at baseline was correlated with baseline vascular function.

CD34 is a surface marker often used in combination with others to define cells as endothelial progenitors, however, it is a non-specific marker of cellular naiveté and CD34⁺ cells are a heterogeneous population. The fact that there was a negative correlation between CD34⁺ cell concentration and baseline arterial function was somewhat unexpected but is in keeping with previous work in which CD34⁺ capture stents lead to an excess of cardiovascular events (Klomp *et al.* 2011).

EOCs represent a more homogeneous population and arguably have a more established role in vascular repair given that they are thought to be the progeny of a true circulating EPC. In this context, the observation that baseline EOC function correlated with baseline arterial function is perhaps not surprising.

This was a small study and the results are hypothesis-generating rather than conclusive but taken together the above findings suggest that the heterogeneous CD34⁺ population widely employed in cardiovascular cell

therapy trials are less relevant to vascular regeneration than EOCs, the *in vitro* function of which correlated with baseline vasomotor function if not vessel recovery.

8.2.3 Late-outgrowth endothelial cells can be cultured using GMP-complaint methods

In vivo study of EOCs in man is contingent upon the ability to isolate and expand these cells using GMP-compliant methods. EOC culture using various products of animal origin is well described (Ingram *et al.* 2004) and a protocol well established in our group. We translated this research protocol for GMP-compliance, removing materials of animal origin.

A GMP-compliant protocol was developed and shown to be effective, reliably producing EOCs which are phenotypically homogeneous, meeting all pre-defined quality control criteria for release, and in sufficient numbers to allow us to consider their use in trials involving human administration.

8.2.4 Late-outgrowth endothelial cells can be labelled using ¹⁸F-Fluorodeoxyglucose without an impact on acute viability or cell function

It is possible that poor cell retention explains the modest treatment effects seen in many cell therapy trials to date (Fisher *et al.* 2015). It is crucial therefore, that prior to moving to a clinical trial of a cellular therapy, one is able to demonstrate

localisation of the administered cells to the proposed site of action and thus bioplausibility. With this in mind, we planned to image administered EOCs with PET-CT using ^{18}F -fluorodeoxyglucose as a label. Crucial steps in this process were to demonstrate not only that EOCs could be effectively labelled with ^{18}F -fluorodeoxyglucose but that there were no acute toxic effect on EOCs.

Optimal conditions for labelling EOCs were defined and assessments of acute viability, proliferation, adhesion and cell cycle were conducted on the labelled cells. Whilst labelling with ^{18}F -FDG caused a dose-dependent reduction in cellular proliferation, acute viability, cellular adhesion and cell cycle analysis were not significantly different between labelled and unlabelled cells. We also demonstrated that sufficient radiotracer was incorporated to allow visualisation by PET-CT and that we could effectively remove unincorporated tracer from the cell suspension following labelling. This will allow us to move to a cell tracking study confident that we are injecting only ^{18}F -FDG associated with cells and that the label has no acute adverse effects.

8.4 LIMITATIONS OF STUDIES

Whilst chapters 6 and 7 could have been improved by more in depth phenotypic analysis of our EOCs during GMP culture and following radiolabelling, the main weakness of this thesis relates to poor reproducibility of the technique of radial artery FMD. A calculation using the mean and standard deviation of the study cohort would suggest that a sample size of 131 would be required to detect a

difference in FMD of 1% with 80% power. Thus whilst able to detect the large change in FMD response seen following transradial cardiac catheterisation, this technique is underpowered to examine subtle differences between subjects and this may have limited the ability to determine subtle influences of EPC populations on vessel function.

Vasomotor dysfunction following cardiac catheterisation and recovery over time was ascribed to endothelial denudation and re-endothelialisation respectively. There was however also a reduction in NMD (endothelium independent) albeit with a different time-course of recovery. Inflammation or injury of the vascular smooth muscle was suggested as the mechanism for this. As the endothelium is the main concern in this model, ideally it would be possible to disentangle endothelial function from vascular smooth muscle function. One possibility would be to use an inhibitor of nitric oxide synthase (which produces nitric oxide in response to endothelial shear stress) such as Methylarginine to demonstrate that the recovery of FMD is abolished when endothelial nitric oxide production is inhibited. This approach would not be capable of demonstrating that dysfunction post-catheterisation is endothelium dependent as smooth muscle (the effector limb of the FMD response) is dysfunctional, but it could demonstrate endothelium-dependence of vessel recovery.

8.4 FUTURE DIRECTIONS

The work presented in this thesis has laid the foundations for a trial of late outgrowth endothelial cell administration and tracking in man which will hopefully lead to further therapeutic trials of these cells.

8.4.1 Tracking late-outgrowth endothelial cells in arterial injury

Late outgrowth endothelial cells likely represent the progeny of a circulating EPC and have several characteristics which make them attractive as a potential cellular therapy, incorporating into host vessels, promoting angiogenesis and attenuating neointima formation in experimental models of arterial injury (Barclay *et al.* 2012; Yoder *et al.* 2007; Patel *et al.* 2016; kang *et al.* 2013 ; Liu *et al.* 2011).

We intend to undertake a tracking study using these cells to document their behaviour in-vivo in man. Thirty patients will be recruited from a stable outpatient population awaiting transradial coronary angiography and they will be split into four study groups as detailed in **Figure 1**.

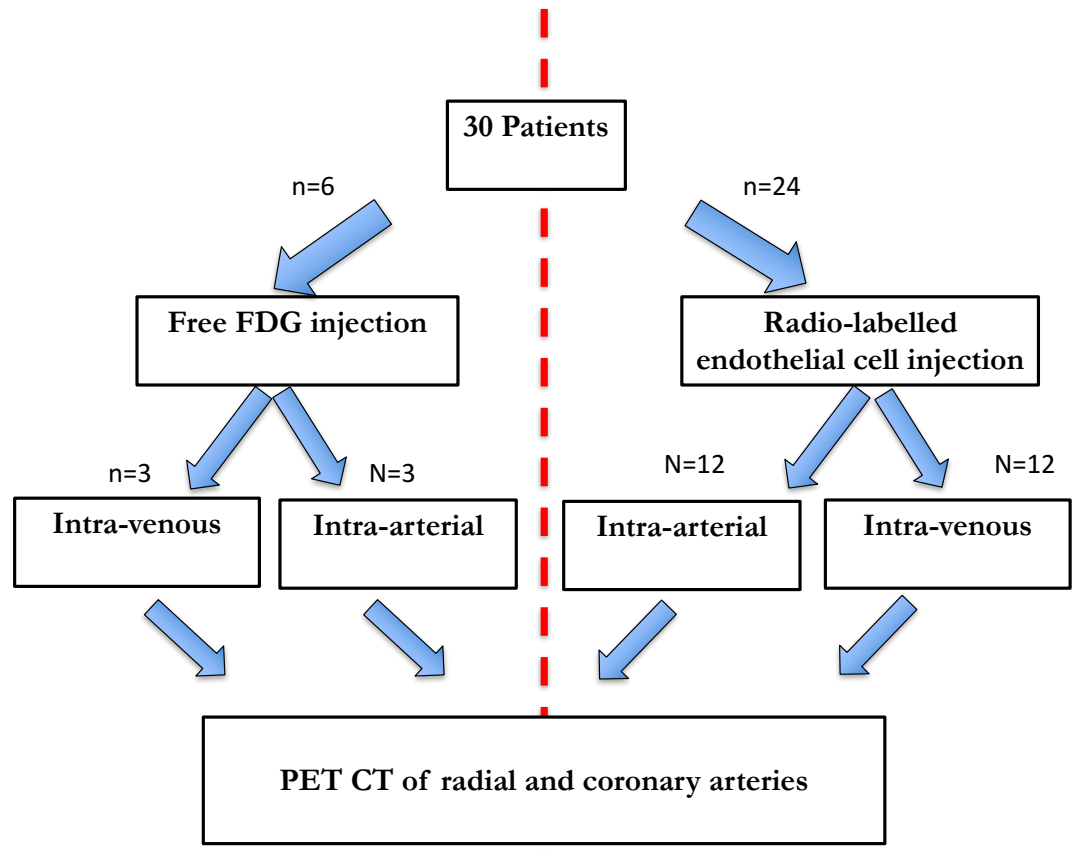


Figure 1: Protocol for ‘Tracking endothelial cells in arterial injury’

Subjects allocated to the cell injection limb will have blood drawn for cell production as previously described, this will be scheduled such that culture day 32 (the final day) falls on the day of angiography. Cells will be released from the cell therapy facility and labelled with ^{18}F -FDG in the hospital radiopharmacy department as described. Free tracer injection will act as a control and will be equivalent to the radioactivity injected with the labelled cells.

Following transradial catheterisation, subjects will be transferred to the imaging facility and positioned on a hybrid PET-CT scanner with their radial

arterial sheath in situ. The field of view will be adjusted to include both radial arteries and the patient's heart. According to study group allocation cells or free ^{18}F -FDG will be injected either intra-venously or intra-arterially via the radial arterial sheath. The arterial sheath will be withdrawn immediately following cell or tracer injection and haemostasis will be achieved using a haemostatic band (TR band™ Terumo, Japan).

Dynamic PET Imaging will then be carried during the 60 minutes from injection and a further late imaging point will assess cellular distribution at 3 h. Study outcomes will be the maximum standard uptake value in the catheterised radial artery and stented segments of coronary artery with comparisons made with the contra-lateral radial artery and non-stented segments of coronary artery respectively, comparisons will also be made between study groups. Dynamic imaging will be used to construct time-activity curves for regions of interest in the radial and coronary arteries to examine the differences in distribution of activity between injections of labelled cells and free ^{18}F -FDG.

Preliminary experience of intra-radial administration of free FDG confirms that we can see a clear signal in the radial artery using 5MBq of which is an approximation of the activity we would anticipate administering which our labelled cells. **Figure 2.**

This study aims to define the biodistribution of EOCs when administered in man and answer the question as to whether they are able to localise to areas of

vascular injury. If this localisation is demonstrated it will provide us with a plausible mechanism by which these cells could affect vascular regeneration and form the basis for therapeutic trials in acute and chronic ischaemic conditions.

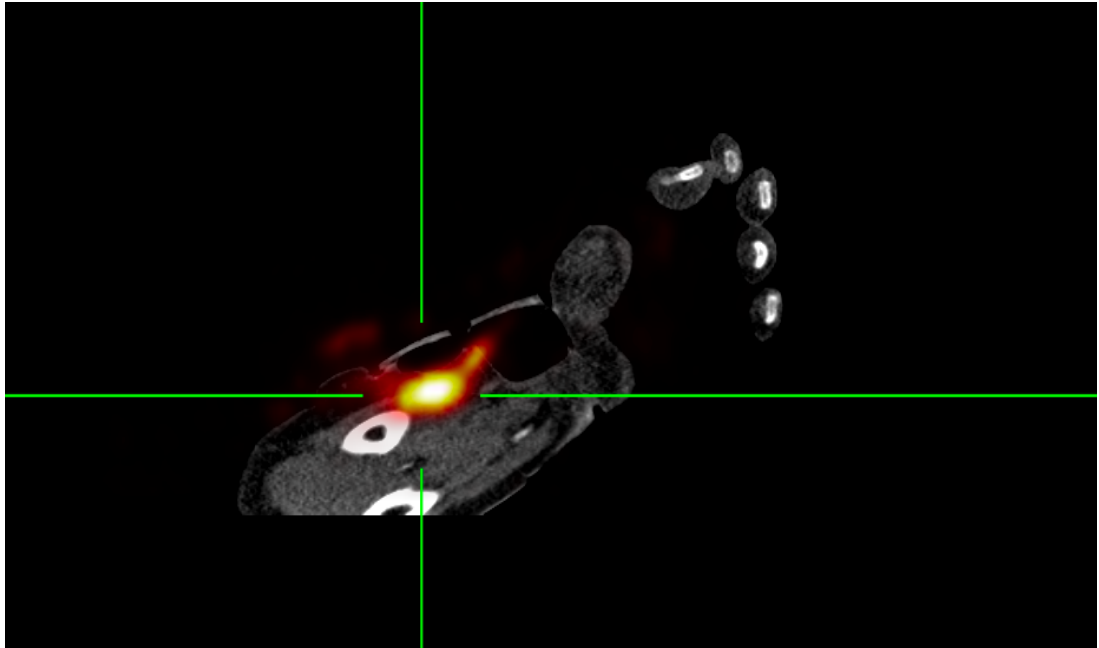


Figure 2: Fused PET-CT image of the radial artery following intra-arterial administration of free FDG (5MBq).

8.4.2 Development of the radial artery as a model of mechanical injury

We studied the radial artery post-catheterisation to examine cellular profiles surrounding vascular injury and to characterise an injury model to be used in future cell tracking studies but there are potential alternatives for the model. The variability of the technique of flow-mediated dilatation (Brook *et al.* 2005; Mitchell *et al.* 2016) makes it difficult to use this model to test therapeutic interventions, although this has been trialled with success in the past (Park *et al.* 2012).

Vascular injury and specifically plaque rupture is a phenomenon of daily relevance to clinical cardiology. Whilst the advent of ultra-high sensitivity troponin assays has dramatically improved our ability to detect myocardial injury (Shah *et al.* 2015), frequent referrals with hyper-troponinaemia of uncertain significance mean that a more specific marker of endothelial disruption and thus plaque rupture would be valuable. Using the radial artery in elective cardiac catheterisation could be a useful model of discrete and defined vascular injury which may be useful in biomarker development.

Intra-vascular imaging of the radial artery with OCT could define the extent of arterial injury sustained at catheterisation (microscopic endothelial disruption could be assumed as a minimum given our experience) and sampling of arterial blood, ipsilateral venous outflow, with comparison to the contra-lateral arms

for levels of putative markers in response to a well-defined vascular injury could be undertaken.

8.4.3 Trials of endothelial outgrowth cells as a therapy

Trials of cell therapy in ischaemic and non-ischaemic cardiomyopathy to date have largely employed either peripheral blood mononuclear cells or CD34⁺ cells, with meta-analyses suggesting a potential benefit but firm conclusion being precluded by imperfect study designs or small sample sizes (Fisher *et al.* 2015; Lu *et al.* 2016). It may be that these cells are not able to differentiate into cardiomyocytes as readily as previously described (Yeh *et al.* 2003) and various less naïve cell populations are currently under trial as regenerative therapies (Sanz-Ruiz *et al.* 2017). Given they represent a more differentiated population, late outgrowth cells may be an agent for myocardial regeneration. Whilst EOCs would not be expected to differentiate into cardiomyocytes, they do incorporate into host vessels and improve perfusion and therefore may support cardiac regeneration, perhaps in combination with cardiac progenitor cells.

Peripheral arterial disease is another area of interest in regenerative medicine and trials, once again largely using peripheral blood mononuclear cells or CD34⁺ cells have generally been more promising than in cardiac regeneration (Rigato *et al.* 2017). Given their convincing capacity to augment angiogenesis in experimental models of limb ischaemia (Goto *et al.* 2016; Minami *et al.* 2015; Dauwe *et al.* 2016) EOCs may be a useful therapy in peripheral vascular disease.

Our GMP-compliant manufacture protocol would allow us to test these cells in man should our initial cell tracking study be successful.

8.5 CLINICAL PERSPECTIVE

Despite the advances made in medical, surgical and percutaneous treatment, cardiovascular diseases continue to cause huge morbidity and mortality. The prospect of regenerating either the vasculature or myocardium is enticing and cellular therapy promises to have a genuine impact on these conditions. To date however, the benefits of these therapies appear marginal and the positive results seen in experimental work have yet to be replicated in the clinical arena.

Cell therapy trials have largely employed non-specific cell populations and it may be that the heterogeneity of the cells administered has reduced the efficacy of these therapies. It is possible that the use of more lineage specific cells than those used to date will be more fruitful. Late-outgrowth endothelial cells are highly proliferative, have been shown to incorporate into host vessels and contribute to angiogenesis in animal models of ischaemia. As such they are a promising potential agent for vascular regeneration in chronic ischaemic conditions.

Given the logistical and financial commitment involved in a clinical trial of a cellular therapy, it will be important to establish biofeasibility by demonstrating localisation of EOCs to areas of vascular injury. By administering radiolabelled

EOCs in the context of transradial cardiac catheterisation we hope to demonstrate homing of these cells to areas of endothelial denudation and confirm that EOCs are indeed a plausible agent for vascular regeneration in man. If shown to home to areas of endothelial disruption in this model, then a therapeutic trial of EOCs is a realistic and logical next step. Whilst re-endothelialisation of the radial artery following cardiac catheterisation is not a clinical problem which needs to be addressed in cell therapy trials, intra-arterial or intra-muscular administration of EOCs in chronic ischaemic disease of the peripheral or coronary circulation may prove fruitful.

In summary, we have demonstrated the utility of the radial artery as a model of vascular injury and established a protocol for culturing and labelling EOCs in a GMP-compliant manner which will allow us to perform cell tracking studies to define their behaviour *in vivo* in man.

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